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Foam Fractionation of Proteins: Concentration of Aqueous Solutions of Bovine Serum Albumin

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Summary. The effects of pH and concentration on foam separation of the protein bovine serum albumin (BSA) from solution has been studied. All results agree, at least qualitatively, with theory. BSA maximum enrichments were observed at the isoelectric pH and enrichment ratio was found to increase with decreasing protein concentrations.

Introduction

Foaming offers a means of separating the components of a liquid mixture when they differ in surface activity. In any such mixture the surface active components are preferentially adsorbed at gas-liquid interfaces while surface inactive components concentrate in the bulk liquid. When the liquid is foamed, the most positively adsorbed (i.e. most surface active) components collect in the foam.[7]

Foaming methods can best be applied to separations involving complex, heat sensitive and chemically unstable materials which cannot be readily or economically separated by the common unit operations—distillation and extraction, for example. Concentration and purification of enzymes, proteins and similar biological materials appear to be good examples of processes to which foaming may be fruitfully applied. The extreme sensitivity of these substances to heat, pH, solvents and other significant variables such as their high molecular weights make these separations very difficult. Special methods such as chromatography, ion exchange, electrophoresis, and isoelectric precipitations have been used but are laborious, require elaborate apparatus and are often unsuitable for final purification.

It has long been known that aqueous protein solutions possess considerable foaming power and yield foams differing in composition from their bulk solutions. Proteins in general yield persistent foams whose stability depends upon pH, the maximum stability usually being observed near the isoelectric point.[3] Although this suggests the use of foaming for the concentration and separation of protein materials, relatively little quantitative work has been reported.

Difficulties inherent in protein analyses have limited many of the protein foaming studies to the investigation of either enzyme systems,[1, 11] which can be analysed by catalytic activity, or entire protein fractions[2, 6] without differentiation between the individual proteins within the fraction.

Theoretical Considerations

GIBBS[9] predicted the increase in concentration of a single surface active material (a solute which lowers the surface tension of the solvent) at a gas-liquid interface. A modified form of the Gibbs relation is given by equation 1:

$$\Gamma = -\frac{X}{RT}\cdot\frac{d\gamma}{dX} \qquad (1)$$

In a two-component system, then, a solute whose addition lowers the surface tension of the solution will concentrate in the surface layer (i.e. in the foam) while a solute which increases surface tension (surface inactive) will concentrate in the bulk liquid.

Extending this analysis to a multi-component solution, the most strongly surface active component (in that particular solution) will have the highest relative adsorption in the foam layer. Unfortunately, no definite rule governing the behaviour of multi-component solutions based upon the properties of solutions of the individual components has been formulated. The effects of a second solute on the surface properties of the solution may be extremely complex, depending not only on solute concentrations and individual solute surface activities but also upon interactions between solutes.

Although equation 1 is the only relationship available for the

prediction of separations attainable by foaming, it cannot be used directly. The concentration term, Γ, is based upon surface area rather than on volume and therefore is directly useful only if a relationship between surface area and volume is known for the foam. Such a relationship is necessarily very complex depending upon the physical properties of the foam solution as well as the mechanical variables of the foaming apparatus (method of foam production, rate of foaming, etc.). Although reasonable estimates of the area-volume relationship can be made, equation 1 predicts only an order of magnitude value for concentration differences between foam and bulk solution.

This equation can, however, predict qualitatively, from surface tension–concentration measurements, whether or not a liquid mixture can be expected to yield such concentration differences. Thus, the Gibbs relation is valuable in indicating when foaming may be fruitfully investigated as a separation method, rather than in predicting the exact values of the separations obtainable.*

In order to achieve separation by foaming, it is not enough that differences in surface activity exist between the components of the solution. The liquid must also be capable of producing a 'stable' foam which will not collapse before it can be collected. Foam stability is dependent upon not only surface tension but also bubble size, temperature, viscosity, pH and other properties of the solution.

Experimental

Apparatus

The foaming column and associated equipment is shown schematically in Fig. 1. Air, at 15 lb/in^2 gauge, is filtered to remove dust and passed through a saturation chamber to prevent subsequent depletion of either foam or bulk by evaporation of water.

Pressure and flow rate are measured and the air stream

* Several reviewers of this paper have commented that the Gibbs relationship is perfectly general and as such can be directly applied, with knowledge of surface tension–concentration relations, to the prediction of surface concentrations. However, it must be pointed out that this statement is true only for a static surface under equilibrium conditions. The foaming operation involves neither static surfaces nor equilibrium conditions; thus the Gibbs equation, even when area to volume relationships are known for the foam, cannot predict exactly the concentration distribution in a foamed system.

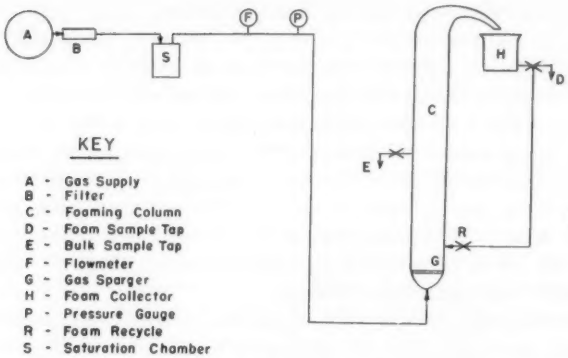

Fig. 1. Schematic diagram of apparatus

introduced to the foaming column through a sintered glass sparger disc. The foam is collected overhead, collapsed and re-cycled to the column to obtain steady state conditions. Foam and bulk liquid samples are removed from sample taps in the foam collector and the column. Physical dimensions of the column are tabulated below.

	Column Dimensions
Overall height	28 in.
Liquid height	25 in.
Column I.D.	$1\frac{9}{16}$ in.
Liquid volume for run	800 cm^3
Sparger disc diameter	$1\frac{3}{8}$ in.
Sparger disc pore size	100 μ
Sparger disc to re-cycle inlet	$2\frac{3}{4}$ in.
Sparger disc to bulk couple tap	$12\frac{1}{4}$ in.
Sparger disc to air inlet	$1\frac{1}{2}$ in.
Foam height	$1\frac{1}{2}$ in.

Experimental procedure

The bovine serum albumin used in this study was obtained from Armour Laboratories, the dextran from Dextran Corporation.

Stock protein solutions containing 0·1 per cent bovine serum albumin (BSA) were prepared initially in distilled water and diluted

as needed. pH adjustments were made with either HCl or NaOH.

Each run was started by charging approximately 850 c.c. of the sample solution to the foam collector and allowing it to flow by gravity through the re-cycle line into the foaming column. The liquid level in the column was maintained constant throughout the run by vertical adjustment of the foam collector. Finally the air supply was turned on and the air rate maintained constant at 77 cm^3/min throughout each 1·5 h run. The temperature was maintained at 28 ± 1 °C. Collapsed foam was continuously recycled from the foam collector back to the column to obtain steady state conditions after which samples of foam and bulk liquid were taken simultaneously for analysis.

Analytical

Protein concentrations were measured on a Beckmann model DU spectrophotometer. Bovine serum albumin exhibits selective light absorption in the region around 280 mμ, the exact position and intensity depending upon pH.[12] Measurements were made at 280 mμ, with the pH of each sample adjusted to 4·7.

Surface tensions were determined with the du Nouy ring tensiometer.[10]

Dextran concentrations were determined by acid hydrolysis (pH = 2) of the sample for 30 min at 250°F followed by sugar analysis using the method of SHAFFER and SOMOGYI.[13]

Results

The results obtained by foaming dilute aqueous solutions of the protein, bovine serum albumin (BSA), are summarized in Fig. 2. Protein concentration in the foam depends upon both the concentration of the bulk liquid and the pH of the solution. Qualitatively, these results are in excellent agreement with theory.

The Gibbs equation, 1, predicts that the ratio of the foam concentration to the bulk concentration (the enrichment ratio) should be proportional to the rate of change of surface tension with concentration in the bulk liquid. From the surface tension–concentration ($\gamma - C_B$) curves for BSA (Fig. 3), it is evident that

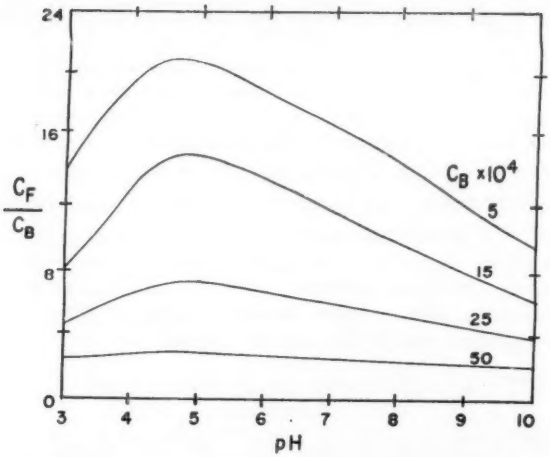

Fig. 2. Enrichment ratio as a function of pH and concentration for BSA solutions

the negative slope of the $\gamma - C_B$ curve for a given bulk liquid concentration is greatest at the isoelectric point (pH of approximately 4·7) with less negative slopes for either higher or lower pH values. Theory would predict from this that maximum enrichments should be obtained at the isoelectric pH. Experimentally, this is exactly what was observed. Similar results have also been reported by LONDON *et al.*[11]

Since a protein exhibits minimum solubility at its isoelectric pH, it is at this point that the protein should have the greatest tendency to collect in regions of the solution where it is least surrounded by the solvent liquid (i.e. at gas-liquid and solid-liquid interfaces). From this, one would expect maximum enrichments to occur at the isoelectric point.

Fig. 3 also shows that the slopes of $\gamma - C_B$ curves increase with a decrease in bulk liquid concentration. At constant pH, then, enrichment ratios should increase with decreasing concentration. Again, experimental results confirm this. (The slopes of the curves of Fig. 3, although apparently zero above bulk concentration

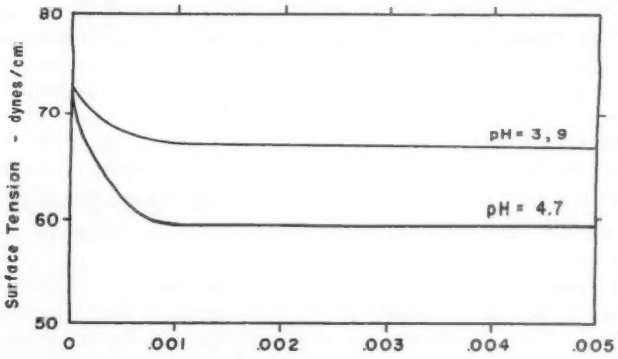

Fig. 3. Surface tension–concentration relations for BSA solutions

values of 0·001 per cent, are actually of finite negative values in this concentration range.)

An exact quantitative comparison of experimental results with theory is impossible at present for several important reasons.

First, the Gibbs equation relates equilibrium concentrations while the experimental results presented here are for steady conditions. In order to estimate exactly how near to equilibrium each run came, data on diffusivity and the activation energy for diffusion are necessary. Unfortunately, this information is not now available; in fact, even the calculation methods are at present uncertain.

Secondly, as noted earlier, the Gibbs equation predicts a foam concentration in terms of surface area rather than volume. Thus, to utilize this equation a relationship between surface area and liquid volume must be known for each foam.

Despite these difficulties, an attempt was made to obtain some quantitative comparison between theory and the experimental results. By assuming that equilibrium was reached and choosing a range for the surface area to liquid volume ratio of 100 to 1,000 cm^2/cm^3, it was possible to obtain a 'theoretical' relationship between the foam and bulk liquid concentrations. Figs. 4a and b compare these 'theoretical' relationships with experimental

results at two different pH values. (The range of foam area to volume ratios used here was chosen using data presented by BIKERMAN[3] for surface to total foam volume and per cent liquid in the foam; 50–100 cm^2/cm^3 surface area per unit of total foam volume, 5–50 per cent liquid in foam).

Examination of Figs. 4a and b shows that the experimental data fall within the range predicted for bulk concentrations above 0·001 per cent. For lower bulk values, the experimentally determined foam concentrations are considerably lower than predicted. This may be due, in part, to experimental error.

A more likely difficulty, however, is the assumption of equilibrium conditions necessary for construction of the 'theoretical' curves. This assumption is surely poorer for the lower concentration range. GAUDIN[8] states that for relatively concentrated solutions, the attainment of equilibrium, primarily dependent on diffusion, can be very rapid; for dilute solutions, the time required for equilibrium is much larger.

Thus, since diffusion rates in dilute protein solutions are low, the contact time between a rising bubble and the bulk liquid will be extremely important in determining the amount of surface active agent which can reach the surface. From this it can be concluded that liquid column height has an important effect upon the separations that can be obtained by foaming. By lengthening liquid column height, contact time between bubbles and liquid can be increased allowing more time for equilibrium near the interface to be approached, and permitting maximum possible separation. Liquid column height will have the greatest effect upon separations for large molecules (such as proteins) whose diffusivities are low.

Foam stability, mentioned earlier, is a necessary requisite for a practical foam separation. In this investigation foam stability was only qualitatively examined. It was noted, however, that stability was highest near the isoelectric pH and at high concentrations, falling off at both high and low pH values and at low concentrations. For the high and low extremes of pH, the minimum bulk concentration which would produce a foam stable enough to be collected was approximately 0·001 per cent BSA by weight; near the isoelectric point this minimum concentration was approximately 0·0002 per cent.

Several runs were made over a wide pH range to determine the

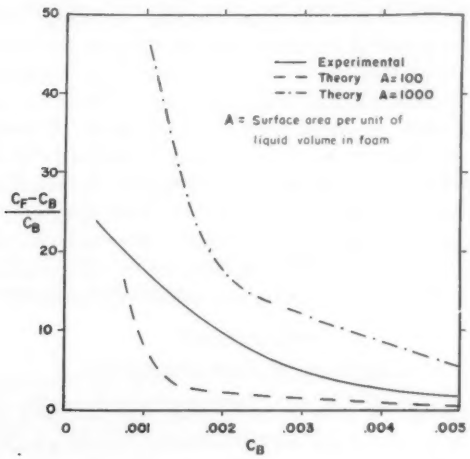

Fig. 4a. Comparison of experimental results with theory pH = 4.7

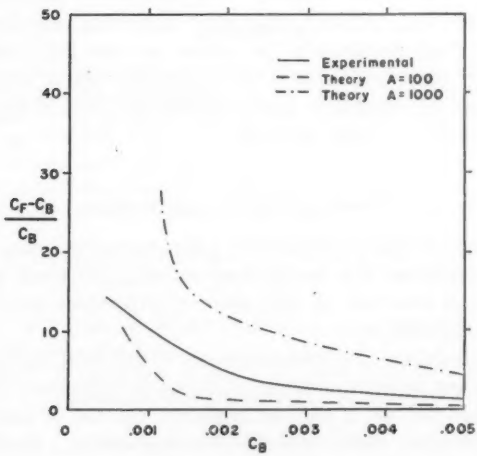

Fig. 4b. Comparison of experimental results with theory pH = 3 or 9

extent of protein denaturation during foaming. Within the limits of experimental accuracy, no detectable denaturation was observed. Since bovine serum albumin is a relatively stable protein, little denaturation was expected. For other, less stable, proteins, however, frequent note has been made of their tendency to undergo denaturation at gas-liquid interface. There is considerable inferential evidence that denaturation at interfaces is as much a chemical effect as it is physical, with degree of denaturation depending upon gas composition. Air, the gas used in this investigation, is moderately active as a denaturant, carbon dioxide more active, and inert gases relatively inactive.

Influence of other components

The final stage of this investigation was devoted to studying the effects of a physically similar second solute on BSA enrichments. The third component chosen was the glucose polymer 'dextran'.[4] The dextran used contained approximately 95 per cent $\alpha - 1$, 6 bonds and had a molecular weight of 104,000. Since dextran is not surface active, no enrichments should be expected when its solutions are foamed. Experimental results bore this out. No dextran enrichments were observed, and the presence of dextran had apparently no effect on the BSA enrichments. From this it can be concluded that a surface active material, such as BSA, can be separated from non-surface active impurities by foaming solutions of the mixture.

Foam separation applications

The results of this investigation point to several uses of foaming in the separation and concentration of individual proteins in solution, and removal of non-protein impurities from otherwise pure protein fractions.

The dependence of enrichments upon pH and surface activity indicates that protein mixtures, whose components differ either in isoelectric pH or in surface activity, can be at least partially fractionated into their individual components. Such mixtures could be most easily separated when the individual proteins differ greatly in surface activity. Only mixtures of proteins having

identical surface activities and isoelectric points would be inseparable.

Since extremely high enrichment ratios are obtained by foaming very dilute protein solutions, a second rather obvious use of foaming would be in preliminary concentration of such dilute solutions.

The third use of protein foaming mentioned above, that of removal of impurities, is similar in principle to the separation of individual proteins. Differences in surface activity between the protein and non-protein impurities would be the basis for separation by foam fractionation.

Nomenclature

Γ = excess solute per unit of surface area—moles/cm^2
R = Universal gas constant—dyne cm/mole °K
T = Absolute temperature—°K
γ = surface tension of solution—dynes/cm
X = solute concentration—weight per cent
C_F = solute concentration in foam—weight per cent
C_B = solute concentration in bulk liquid—weight per cent
C_F/C_B = solute enrichment ratio
A = surface area per unit of liquid volume in foam—cm^2/cm^3

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The Large-Scale Separation of Fluids from Fibrous Pulps

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Summary. Most of the free fluid can generally be expressed from fibrous pulps in a few seconds at 50 lb/in^2 if the layer of pulp is kept thinner than 1 in. after it has been pressed, and if the fluid is allowed to run away freely. The need for this time and the frictional properties of many pulps are serious obstacles to the design of any strictly continuous press. Roller presses do not allow enough time, screw expellers create too much friction, continuous centrifuges and some other arrangements are intricate and vulnerable.

An intermittent but automatic press which avoids these defects is described. In this the material on a perforated conveyor is pressed by a ram and then moved forward as the ram lifts. One such machine has already been made. An outline is given of the principles on which an improved design will depend.

Introduction

On a small scale the simplest way to separate fluid from a fibrous pulp is to pick up a handful and squeeze it. This illustrates two important points in the process. With many pulps, the mass of fibre left in the hand, though still moist, does not lose much more fluid if subjected to very much greater pressure: the particles carried out into the fluid are generally much smaller than the gaps between one's closing fingers because the fibre itself acts as an efficient strainer. This means in practice that much of the fluid comes away with pressures only rising to 50 lb/in^2 and that there may be no need to have a fine screen. It is generally easier to allow a little fibre to escape in the first stage and then separate it by a second stage of filtration rather than to try to get complete separation in one operation. Although the operation appears to be very simple, and although it is probably as old as agriculture, it is surprisingly difficult to carry out satisfactorily on a large scale, yet there is an increasing demand for the necessary

equipment. As examples, the de-watering of various textile fibres, fungal residues, peat, and fodders that have been steamed or in some similar way robbed of the ability to hold water osmotically, may be quoted. Most of my own experience has been gained in the course of making leaf protein as a food for man and other non-ruminants (PIRIE, 1942, 1952, 1957). This application raises some special difficulties. The fibre is harsh and unlubricated, the juice is of low commercial value, and much of the protein sought is in the form of intact or damaged chloroplasts and other subcellular particles with diameters from 0·1 to 10 μ. These are filtered off and retained by the fibre if an attempt is made to force fluid through a compacted mass. This is easily demonstrated in the laboratory by comparing the protein content of successive fractions running from a press filled with pulped leaves. The first fractions may contain three times as much as the final fractions coming out after the fibres have been tightly packed so that they begin to act as a semi-permeable membrane.

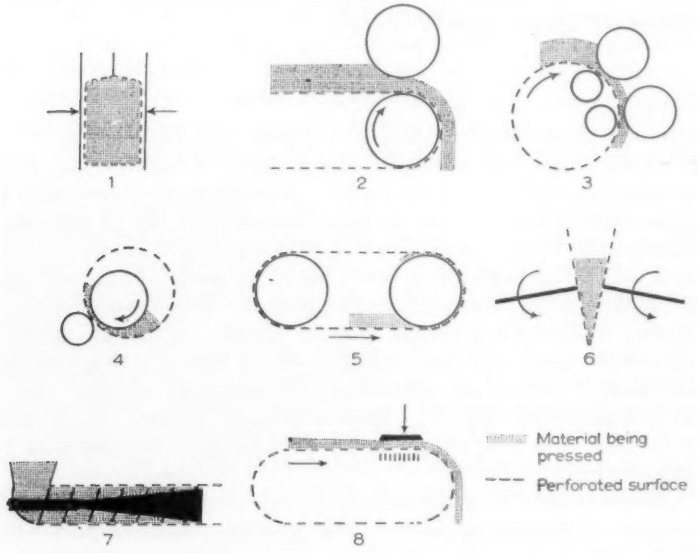
With these considerations in mind, some techniques will be discussed that are applicable to pulps containing initially 80–90 per cent of fluid of which 50–90 per cent can be removed by pressure alone. The methods used when the starting material is wetter, as in mineral dressing and paper making, involve different problems which have already been fairly satisfactorily solved. Evaporative removal of water is an entirely separate problem.

The amount of water in most samples of pulped leaves is insufficient to allow any significant amount of juice to run out when the mass is simply put on a sieve ; shaking does not bring out much more. Suction, that is to say the application of up to 14 lb/in^2 to the surface exposed to the atmosphere, forces much of the juice out but after this pressure the cake is very moist and constant attention is needed to fill up the fissures that develop which would make suction inefficient. All practical methods therefore depend on the application of pressure to a constrained mass of pulp and the variations are restricted to the manner in which the pressure is applied.

Pressure on a bag

The simplest arrangement is a fabric bag to which pressure is applied directly or by twisting (Fig. 1). This is a common and

satisfactory laboratory technique and it is still used on a large scale in making glue and in a few other industries. Some filter presses depend on essentially the same mechanism; a row of bags hangs on a rack and the whole row is pressed from one end. Or the charge is pumped into the space between fixed fabric-covered leaves. Not only is much labour consumed in filling and emptying



Figs. 1–8. Techniques used in separating fluids from fibrous pulps.

the units but, for the reasons already given, the composition of the liquor varies greatly during the course of one pressing. The method is therefore satisfactory on a small scale or when the particles being extracted are very small, but it is inconvenient on a large scale or with larger particles.

Rollers and a Conveyor

The disadvantages of pressing in a bag can be avoided to some extent if the charge is laid out on an endless belt which is then

fed through the nip of one or several pairs of rolls. The action is now continuous, the charge can be kept thin, it is to some extent re-arranged after each application of pressure and the re-arrangement can be made thorough if need be, and water can be added to increase the efficiency of extraction without breaking the continuity of the process. Processes of this type have been used by many different people with many different materials but never apparently wholly satisfactorily.

Flexible Conveyor

In the paper industry, fabric or woven wire belts are used to carry the paper pulp through various stages of de-watering, first by straining and then between rolls. A machine developed from a paper-making machine ran for some years in the City of Birmingham Salvage Department, pressing the water out of ground household waste which was then dried for cattle feed. In this design the ground material went through a pair of rollers on to a felt that carried it through a second pair of rollers. Although various difficulties arose with this arrangement so that it was given up, we made a similar machine from two domestic mangles and a loop of sackcloth (Fig. 2). Several hundred gallons of leaf juice were made with this, but it was far from being an ideal solution to the problem.

One objection to this principle is the vulnerability of the belt, another is the short duration of the pressure. In principle a rigid body passing through rigid rolls is pressed for an instant only; in practice the compressibility of all surfaces extends the pressing period but even with very slow moving rolls it is difficult to extend it to more than a second. Experience shows that at least five seconds is needed to allow the juice to flow so far out of the fibre as to avoid being sucked in again as soon as the pressure is relaxed. The first difficulty has been met in the 'Louisville Dryer' by replacing the fabric belt by a belt made of a series of perforated metal plates hinged together. The Madruck press meets the second by passing two similar belts between many pairs of rolls set sufficiently near together to allow the rigidity of the belt to maintain some pressure between nips. This press is extensively used on peat in Germany and Scandinavia; the usual

models are therefore very large. The principle could probably be adapted to pressing leaf pulp but this has not yet been done.

Rigid Conveyor

In these presses the flexibility of the conveyor is a source of difficulty. A rigid conveyor in the form of a cylinder can clearly be used to go through several pairs of rolls so long as they are set on the arc of a circle (Fig. 3). A press of this type was made by the National Institute for Agricultural Engineering (1954) and worked fairly satisfactorily on leaf pulp for some years. As in all machines that apply pressure with rollers in this way, the duration of pressing was too brief; this was the only serious defect in the press.

For a given peripheral velocity, the duration of pressure increases as the diameter of the rolls applying it increases. A still further increase is obviously possible if the pulp to be pressed is fed on to the inside of the conveyor so that it is pressed between the conveyor and the inner roll (Fig. 4). This is the principle of the 'Hornangle' or 'California' press and it has been applied by Stacom (1953) to the expression of fluids. It will not be altogether easy to get the rather intractable pulp from leaves into the inside of the conveying roll and to spread it in an even bed, nor will it be as easy to get the pressed material out of the system as it is to get it off the upper surface of a conveyor; but neither difficulty should be insuperable.

Variants on the Roller Principles

In the last arrangement an internal roll presses against a rigid conveyor that is supported by a roll or rollers outside. These need not necessarily be opposite the internal roller provided the conveyor is strong enough. This leads naturally to the idea of a flexible perforated conveyor held in tension by two internal rollers exactly as in a flat-belt pulley drive (Fig. 5). The material would then be under pressure during 180° travel round the roller. A machine on that principle has not, apparently, been made but there is no obvious objection to it, and the problems of filling and emptying are made easier by the wide separation possible between the two rollers.

An alternative method of getting prolonged pressure in a continuous machine is used in the 'P & L De-watering Press'. Here two perforated discs are set face to face on shafts that meet with their axes at a slight angle to one another (Fig. 6). The space between the discs is therefore wider at one side than the other. Both are driven and pulp introduced at the wide point is pressed when carried through the narrow part, and removed as the space opens again. Although this arrangement has not been tested on pulped leaves there is good reason to think that it would be satisfactory.

Screw Expellers

There are many different designs of screw expeller but all depend on the same basic principle. The material to be pressed is forced into and through a perforated chamber by helical scrolls carried on one or more shafts (Fig. 7). The volume swept out by an element of the scroll during a revolution is greater at the feeding than at the discharge end, and the ratio of these two volumes is so adjusted that the required pressure is built up. A screw only acts as a conveyor when there is relative motion between the scroll and the material being conveyed. Thus anything that remains attached to the scroll simply goes round in the same plane for the same reason that a wood screw does not penetrate a block of wood that is itself free to rotate. The action therefore depends on the whole of the front face of the scroll being pressed against the charge and rotating against it; in so far as this does not happen, so that the charge is able to rotate too, the expeller is being inefficient. Existing machines are no doubt satisfactory under the well lubricated conditions of oil expulsion but friction makes them extravagant of power when an aqueous juice is being forced out from a harsh fibrous pulp. This is not only wasteful but the power appears as heat in the charge and this may be detrimental; it also leads to further sub-division of the particles in the pulp so that the juice is contaminated with finely divided fibre. To make sure that these disadvantages do not exist in theory only, a dozen different types of expeller have been tested on pulped leaves during the last 18 years. They work after a fashion but none has proved satisfactory.

It would be possible to get rather better performance by modify-

ing the usual expeller design in a few particulars. Thus pressure should not be built up by closing the outlet by a gate or other obstruction because this leads to too sudden an increase in pressure at the very end of the machine. There should instead be sufficient difference between the volumes swept out at the two ends of the screw, by an element of the scroll, to cope with the maximum diminution in volume that is likely to be met during the pressing of any pulp. This diminution varies with different materials so that adjustment is necessary, and this can best be got either by keeping the outside diameter of the screw constant and sliding it into different positions in the casing, as is done in some expellers, or, more cheaply, by having removable elements at the high-pressure end of the casing so that the fibre can be discharged at the point at which the right ratio is reached. In many expellers the diminution in volume is achieved by shortening the pitch of the screw while keeping the diameter of the central spindle or hub substantially unaltered. This means that juice has to penetrate through as thick a layer of packed fibre at the high- as at the low-pressure end, which is a mistake. It is much better to keep the pitch substantially constant and increase the diameter of the hub; this is an especially important point when useful components of the juice are present as particles in the 1–10 μ size range, for these are increasingly likely to be retained as the depth of filtering material is increased. Even with these improvements in design, however, friction will continue to limit the range of applicability of screw expellers.

Centrifuges

The basket centrifuge is an excellent device for separating juice from fibre on a small scale. In it the juice does not run out because it is all under hydrostatic pressure, as in a press. Separation does not therefore stop as soon as the volume containing the solid phase has been so much diminished that the solid is itself supporting the load that produces the pressure. Instead, each separate droplet of liquid is exposed to an individual outward acceleration regardless of the behaviour of the others. This is especially important with relatively undeformable fibres, for with them pressure alone will not get the fluid out from the spaces that the fibres do not fill, but the fluid can be centrifuged out. Thus the void left

among equal spheres varies from 0·48 to 0·26 according to whether the spheres are packed in the loosest (cubic) or the tightest (rhombohedral) array, and this void would be left filled with fluid if a suspension of these spheres were put in a press that did not deform the spheres. The void also would be emptied in a basket centrifuge.

Various arrangements are used to get continuous operation. A ram at the inside end of the bowl of a basket centrifuge can push the whole charge forward intermittently, or a plough can scrape it out, or a screw conveyor running at a slightly different speed from the basket can move it steadily along from the filling to the discharge end. These arrangements are satisfactory with many materials but a centrifuge so equipped is intricate and expensive and the propelling mechanism has difficulty in moving an unlubricated fibrous mass that tends to felt together.

Conveyor and Ram Presses

From this examination of the action of various common types of press several principles emerge.

(1) Continuity of action is an advantage but it is not an essential feature so long as an intermittently acting press is automatic and involves no manual work. The pursuit of continuity has introduced difficulties; thus the charge is moved through the press while under load in the Madruck press, and it is even moved relative to the filter surface, while being pressed against it, in expellers and centrifuges.

(2) The thinner the layer of material being pressed the better, but the thinner the layer the smaller the throughput. Other things being equal, throughput is directly proportional to thickness, whereas each successive increment in thickness obstructs outflow more than the last. In practice the layer of material, after pressing, should not be more than 1 in. thick.

(3) The total amount of juice expressed depends on the pressure but, provided time is allowed for the juice to run away, the advantages of going beyond 50 lb/in^2 are out-weighed by the increased stresses on the machine.

(4) Because of the deformability of most fibres, the outflow of juice does not stop suddenly but, at any pressure, there is sufficient

re-arrangement of the pressed fibre to keep up a gradually diminishing outflow. In practice at least 90 per cent of the juice that is already free in a pulp will come away in a few seconds; but it will not come away in a fraction of a second so long as gravity is relied on to remove it from the filter surface. The time that it takes a drop to flow or fall from a filter surface could be shortened by devices to suck or blow it away, or by increasing the gravitational field as in a centrifuge, but these are serious added-complications. It seems simpler to accept the necessity for some delay and adjust the pressure accordingly.

With these considerations in mind a conveyor press was made in which an endless belt made of hinged perforated plates passes under a ram (Figs. 8–10). Pulp is fed on to the upper horizontal surface of the belt. The ram is driven by a double acting hydraulic cylinder connected to timing gear and valves so that it can be set to press down for 2–100 sec and then lift in less than 1 sec. During the lifting stroke a ratchet arrangement moves the conveyor forward and brings a fresh portion of pulp under the ram. Although this intermittent action seems inefficient, because the conveyor is constantly being stopped and started, the inefficiency is more apparent than real because the work is done at a period when the motor would otherwise be idling. The perforated part of the conveyor is 12 in. wide; as a rule the machine is set to take a bed of pulp 2 in. deep, press it for 10 sec and then advance the conveyor 10 in. It should therefore press nearly 1·5 tons of pulp an hour. In practice it is wise not to use the full width because the pulp splays out a little as the pressure comes on and the bed is not altogether uniform but has incompletely filled patches in it. The usual rate of working is therefore half to three-quarters of a ton an hour. For this only 1 h.p. is needed. The pressed fibre still contains 65–70 per cent of water. It can be made a little drier by pressing for more than 10 sec but the consequent diminution in throughput does not justify prolonged pressing. In practice it is nearly always more economical to pass the fibre, with added water, through the pulper a second time, and so recover not only the juice already liberated but not expressed but also more juice that is liberated during the second pulping. Two photographs of this machine are shown in Figs. 9 and 10 (PIRIE, 1957).

Many tons of leaf pulp have been pressed with this machine and

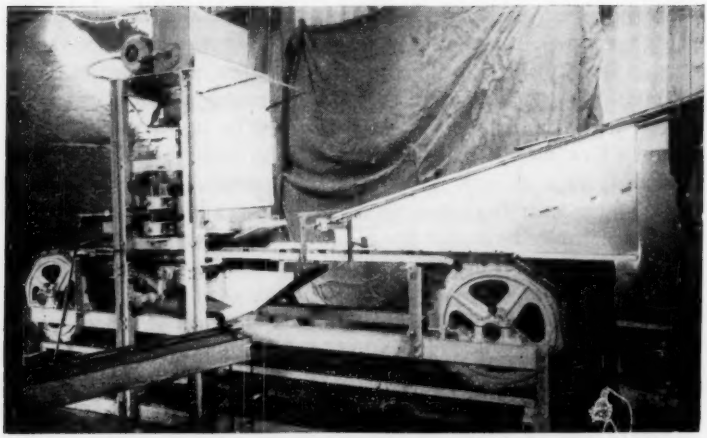


Fig. 9. General view of the press. Pulp is thrown on to it under the cowl on the right. The perforated conveyor moves counterclockwise intermittently under a hydraulic ram towards the left. The juice comes out near the middle and fibre falls off at the extreme left.

it is unquestionably more satisfactory with this material than any other press or expeller that has been tried. Difficulties arise with two types of material. Pulp that is slippery, for example leaf fibre that has been treated with alkali, has some tendency to slip sideways out from under the ram. This could of course be overcome by applying pressure to the sides first or by containing the material as it is being pressed, but this involves complications of the machine and the tendency to slip may be taken as evidence that material like that would be handled satisfactorily by a screw expeller. Pulps containing very little fibre or very short fibres also cause trouble because the whole pulp tends to be pressed through the conveyor even when the perforations are only $\frac{1}{32}$ in. wide. Fabric stretched over the conveyor improves the performance but is troublesome and difficult to clean. If material with this consistency has to be pressed it is probably better to do it in a filter press, but the consistency can often be altered without detriment. Thus sugar beet leaves disintegrate on pulping into an intractable *puree* and autumn grass is sometimes slimy and

Fig. 10. A view of the press from the discharge end showing the ram lifted and the hydraulic cylinder that actuates it.

gives poor protein extraction. A mixture of the two, on the other hand, is a very satisfactory material both from the standpoint of protein extraction and of behaviour on the press.

Nevertheless, the existing press has defects. The need for a flexible conveyor complicates design and greatly increases the weight of the whole machine. The conveyor does not have to withstand the pressure of the ram because at the point where this is applied it is adequately supported by a robust grid; in principle therefore a fabric conveyor should suffice instead of perforated plates. In practice fabrics get clogged with fibrous material and, though satisfactory with the light pressures used in paper making, they are less suitable than perforated metal in this type of press.

It would therefore be better to use as a conveyor a rigid annulus of perforated metal instead of a hinged belt. This would be mounted with its plane horizontal so that the return route of the conveyor would be on the same level as the part where pressure is applied instead of underneath it.

The ease with which the intensity and duration of pressure can be varied when pressure is applied by a hydraulic cylinder and timer are a great advantage in an experimental machine; but they are an unnecessary refinement once the conditions for satisfactory pressing have been found. It would then be simpler to apply pressure by means of a large slow-moving cam with a profile adapted to the required rhythm of pressing and then lifting the pressure plate. A press embodying these principles is now being made. It is so designed that the conveying annulus can be easily removed and replaced by others with different perforation patterns, and the cam can be replaced by others giving a different rate of application of pressure. The duration of pressure will be varied by having alternative V-belt pulleys connecting the motor to the input side of the reduction gear that drives the camshaft. No doubt difficulties will be discovered in this press too when it comes to be used, but it is probable that it will have a wider range of applicability than any of the presses that have so far been tried.

Every problem of juice expression is in some respects a special problem so that it is unlikely that one machine will suit all materials. The conveyor and ram principle is not therefore put forward as a general solution to the problem of getting fluids from fibrous pulps, but it has the merit that there is no relative motion between the press surfaces and the material being pressed while pressure is applied, and there are many applications where this feature would be valuable.

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Equipment for Cultivation of Fastidious Micro-organisms

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Summary. Equipment has been developed in which fermentation processes for fastidious, pathogenic micro-organisms can be operated on a pilot-plant scale. Experience has shown the need for the development of concepts, techniques, and equipment meeting more stringent requirements than those ordinarily encountered in the fermentation industry. A system has been developed for the preparation of pure cultures which may be used either for the development of processes or in the preparation of substantial quantities of pure cultures. The equipment includes specially designed valves, fermentors, piping arrangement, and requires special methods of operation in order to maintain pure cultures during growth of the organism. The number of cultures of pathogenic, fastidious micro-organisms contaminated in the systems commonly used in industry is materially greater than the number of contaminated cultures produced in the system described.

Introduction

Specially designed equipment is called for in studying the bio-engineering aspects of submerged fermentation processes involving fastidious pathogenic micro-organisms. Two major problems confront the designer of fermentation equipment for this purpose: (1) prevention of entry of contaminant organisms into the system to allow production of a pure culture of the organism under study; and (2) prevention of the escape of pathogens from the equipment to assure safety of operating personnel. The problems involved in the preparation of pure cultures of this

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type arise from the fact that conditions required for the growth of pathogenic organisms are generally highly satisfactory for the growth of contaminants; the generation time of most pathogens is longer than it is for most contaminants, and most pathogens do not produce by-products which will retard or prevent the growth of contaminants.

Past experience in the design of equipment for producing pure cultures of fastidious organisms has demonstrated the need for more refined techniques than those commonly employed by the fermentation industry. Designs similar to those used for production of alcohol and antibiotics were tested and found to be inadequate. A large percentage of the cultures prepared in this type of equipment became contaminated to a point where the data obtained could not be considered reliable.

The results of investigation into the causes of contamination indicated that the systems could not always be sterilized with either liquid decontaminants or steam at 40 lb/in^2 gauge. The primary sources of contamination, after attempted sterilization, were valves, pockets or crevices, packing glands, and flanges; also, the isolation of the fermentor unit was found necessary in order to prevent cross-contamination between units.

Equipment

A system was developed, and is shown diagrammatically in Fig. 1, in which pure cultures can be produced 95 per cent of the time without exposing operating personnel to undue hazards. The important features of this system are the designs of the fermentor, valves, piping arrangement, sampling adaptor, and the use of a steam seal tank to isolate the fermentor from other equipment in a plant, and to eliminate these sources of contamination.

The fermentor is an all-welded vessel, constructed of stabilized stainless steel. Stabilized steel is required in order to prevent intergranular corrosion near welds which cannot be satisfactorily heat-treated. Intergranular corrosion results from the precipitation of chromium carbide in the grain boundaries, causing a depletion of chromium in the area of welds. Fermentors which were constructed of non-stabilized steel corroded during fermentation (at pH 6·8 to 7·8) and small, pin-hole leaks developed.

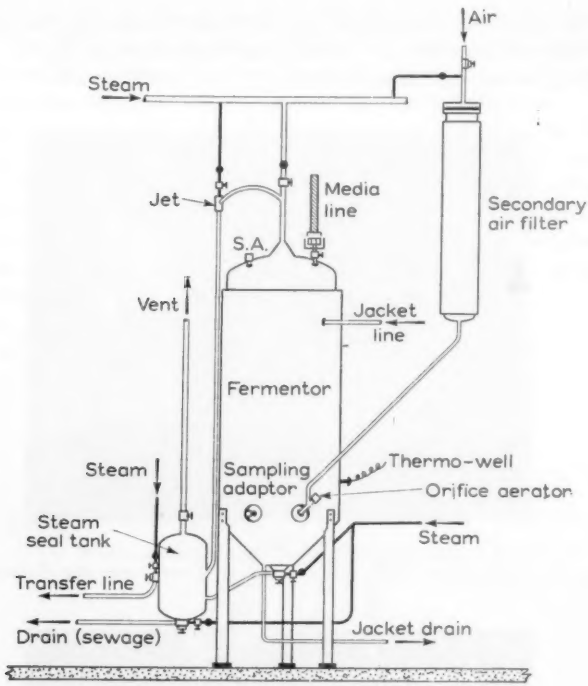

Fig. 1. Pure culture system

All welds in the equipment are ground smooth, and the surfaces of the fermentor which come in contact with the culture are polished to a number 4 finish for ease of cleaning. There are no flanged openings in the vessel which allow hand cleaning. The tanks are washed periodically with hot, 5 per cent caustic solution. A fermentor of this type was used to prepare numerous cultures of various organisms over a period of six years with routine washing. Then, the tank was opened to determine its condition prior to a routine washing and to evaluate the effectiveness of the simple washing technique. The appearance of the fermentor interior after removal of the dished head is shown in Fig. 2. This fermentor was cleaned by filling the tank with hot, 5 per cent

caustic solution, mixing with air, and then removing the solution through the drain valve. Results are illustrated in Fig. 3. There is no apparent corrosion and the finish is still smooth.

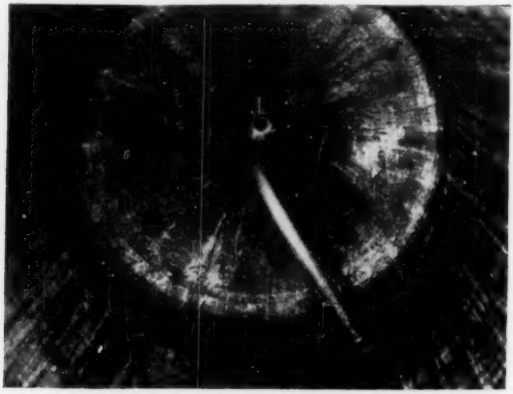

Fig. 2. Fermentor No. 4 immediately after removing the dished head

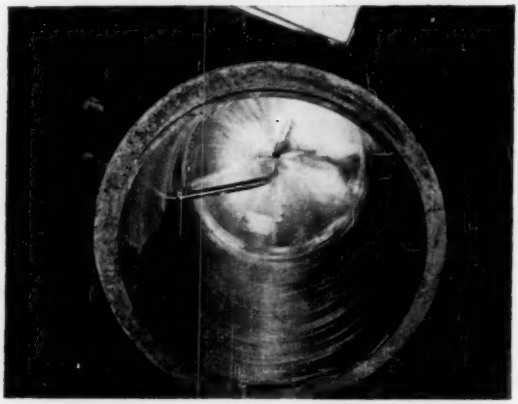

Fig. 3. Fermentor No. 4 after cleaning by caustic washing, without scrubbing

The secondary air filter was designed as an integral part of the unit. The filter consists of a column of glass wool which is maintained, by means of an insulated steam jacket, at a temperature above the dew point of the air passing through the filter. The filter is operated with the air passing *down* through the filter material in order to eliminate pockets in the air system and to isolate flanges from the fermentor. All water vapour passing through the filter is prevented from condensing by the controlled temperature. This is necessary to prevent condensation of moisture which would flow by gravity through the filter, carrying contaminants into the fermentor.

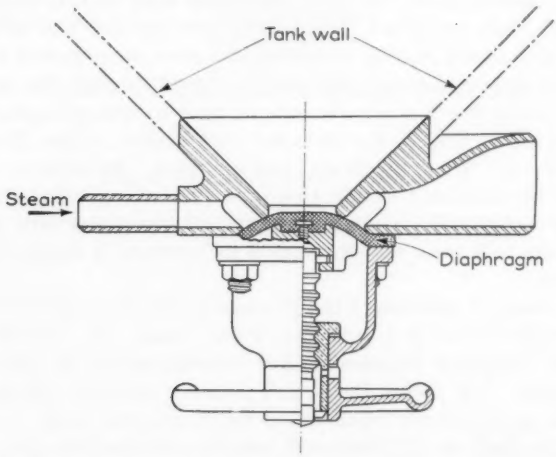

Fig. 4. Fermentor dump valve

The bottom dump valve is an adaptation of the Saunders valve and is an integral part of the vessel. A diagrammatic sketch of the valve is shown in Fig. 4. The diaphragm sits against the apex of the cone bottom of the tank, thus eliminating the pocket which would be formed by a pipe stub. During culturing, low-pressure steam is passed around the top of the diaphragm and out through the product port into a small steam seal tank, in order to provide a steam seal between the valve and other equipment.

Saunders diaphragm valves are used in all locations which come in contact with the organism under study. Gases are removed from the fermentor, through a vent line in the dished head, and passed through a steam jet. The mixture of gas and steam is exhausted into the steam seal tank. The action of the jet maintains a negative pressure of approximately 6 in. of water in the air void above the culture during fermentation.

The negative pressure assures that any leakage which may occur in the head of the vessel is internal, thus reducing the hazard to operating personnel. The steam seal tank provides a means of isolating the equipment from other parts of the system. During operation of the unit, the steam seal tank is constantly supplied with saturated steam from the jet and the fermentor drain valve steam supply. Vapour and gases are removed through the vent system; condensate being removed through the partially opened drain valve of the steam seal tank to sewage equipment.

As noted in Fig. 1, the installed pipe system of the fermentor includes only the vent, drain, and air lines. In order to contaminate the culture through this pipe system, an organism must either pass through the air-filtering system or counter-flow through the steam seal tank and through lines containing steam into the fermentor.

The internal surfaces of the processing line are smooth and free from irregularities (e.g. crevices, welds, beads, etc.) which might provide stagnant lodgement for contaminating or pathogenic organisms. All welds for which smooth interior surfaces are difficult to obtain are localized on the steam seal tank.

Sterile medium is introduced into the system through a stainless steel line which is connected to the vessel by means of a ground-faced coupling. The connection is made prior to sterilization and transfer of medium, but is disconnected during growth of the culture. After the diaphragm valve at the tank is closed, the line is disconnected; the nozzle is capped and flooded with a decontaminating fluid by filling the reservoir surrounding the male half of the coupling.

Materials such as inoculum and antifoam are introduced into the tank, and samples are removed by way of a sampling-adaptor assembly in the side of the fermentor. The adaptor consists of the body, which is welded to the vessel, a rubber diaphragm, a

metal washer, a metal retaining ring, and a removable cover cap. Fig. 5 shows the relative positions of these parts in the completed sampling-adaptor assembly. Entry is made by puncturing the self-sealing rubber diaphragm with a hypodermic needle. The sampling adaptor in the head of the vessel is used to introduce a

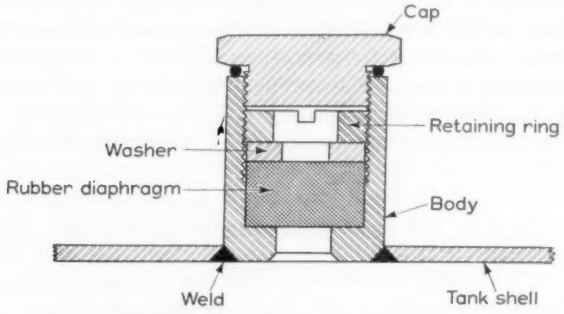

Fig. 5. Sampling adaptor assembly

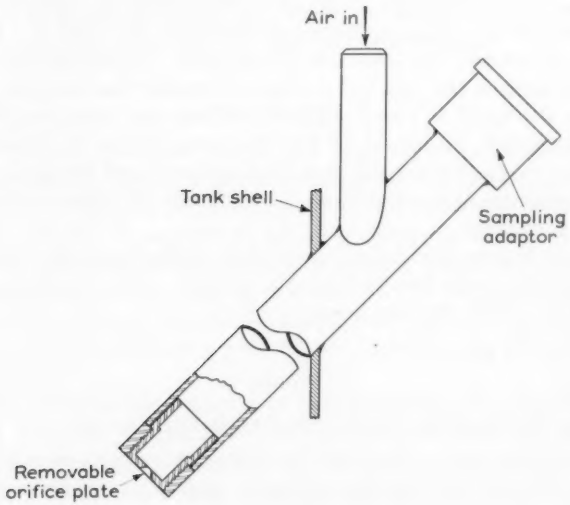

Fig. 6. Orifice aerator

probe for foam detection and it may also be used for introducing antifoam or inoculum.

The culture is aerated by passing sterile air through an orifice, submerged in the culture, at critical pressure ratio, as described by ACHORN and SCHWAB.[1] The orifice aerator, Fig. 6, is welded in the fermentor at an angle which assures that all liquid in the line will remain within the fermentor. The assembly is composed of three main parts, the air inlet pipe, a sampling adaptor, and a removable orifice plate. The orifice plate has key slots to permit removal for cleaning or replacement. The orifice plate is withdrawn or inserted through the sampling adaptor at the upstream end of the pipe.

Operation

Sterilization

The system is sterilized with steam as follows:

The media line is connected to the fermentor and the media valve as well as the dump valve of the fermentor are opened. The vent and drain valves of the steam seal tank are opened. Steam is introduced into the jacket of the air filter. Steam is introduced into the system through the air line, the media line and the steam line to the head of the fermentor. When the temperature has reached 212°F, measured by the thermocouple in the fermentor, the vent valve of the steam seal tank is closed and the drain valve is adjusted to allow condensate to drain but to maintain pressure in the fermentor.

Steam is introduced into the jacket of the fermentor, and the valve in the steam line to the jet is opened. The system is maintained at 250°F for two hours.

Cooling

After sterilization, the system is cooled as follows:

The drain valve of the steam seal tank is closed. All steam valves, except those to the air filter jacket are closed and air is introduced into the fermentor to maintain pressure. Cold water is then circulated through the fermentor jacket. After the tempera-

ture is reduced to the desired level, it is regulated by means of temperature control of the water circulating through the fermentor jacket.

Fermentation

After cooling, the system is prepared for inoculation:

The fermentor dump valve is closed and the steam line to this valve is opened. The steam seal tank vent is opened and the drain valve is partially opened. Steam is introduced to the steam jet and the supply of air is stopped. The desired volume of sterile medium is passed into the fermentor through the media line. The media valve is closed; the line is disconnected, and the male portion of the coupling is capped and flooded with liquid decontaminant. Medium is inoculated through one of the sampling adaptors with a hypodermic needle. Air is introduced into the culture at a controlled pressure through the orifice.

After completion of fermentation, the culture may be removed by way of the steam seal tank either to the sewage system or to other equipment. The system is then sterilized as described above. It is not necessary to attach the media line in order to sterilize the fermentor and the installed pipe system.

Conclusions

The system described herein has been used for studying the bio-engineering aspects of the fermentation processes of fastidious organisms. Cultures with characteristics similar to those prepared in laboratory equipment have been prepared in this system. Aeration efficiency of the fermentor, using a single orifice of the type described in this paper to introduce 0·375 volume of air per volume of culture per min, is 0·7 mMO_2 per l. per min on the basis of the sulphite oxidation procedure used by COOPER, FERNSTROM and MILLER.[2]

Higher aeration efficiencies may be obtained by use of additional orifices or by introducing the air tangentially to the vessel. Little difficulty was encountered with this type of system with respect to contamination or safety for operating personnel. When the system did become contaminated, the two-hour sterilization procedure described was adequate for complete decontamination. In

one test the system was contaminated with a bacteriophage for the strain or organism under study, resulting in complete destruction of the culture. The system was sterilized for two hours as described above and then used to continue studies with the same strain of organism. No further difficulty was encountered, indicating that the phage had been destroyed.

Acknowledgement

The equipment described herein resulted from the efforts of personnel of Fort Detrick, Frederick, Maryland.

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The Significance of Surface Electric Charge on Algae in Water Purification

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Summary. The results of a study on the surface electric charge carried by fresh-water planktonic algae are used to provide a theory for the flocculation of algae with chemical coagulants. Where it is desirable to remove algae from water, for example from lakes or reservoirs for water supply, or from sewage oxidation ponds, the theory indicates certain practices are desirable to obtain the best removal. It is planned to extend the theory and experimental work to cover the removal of untreated algae from water by filtration through porous granular media, believed to be an adsorptive effect. Separation of different species of algae employing their electrokinetic properties is also described.

Introduction

Only recently was it established that planktonic algae in water carried an electric charge on their surfaces. Previously it was well known that silt and other inorganic suspensions exhibited a negative electric charge, and in addition that bacteria also carry a charge. That it should be so for the relatively larger algae (size range 5–50 μ) was guessed, but not proved experimentally until 1955.

The algae selected for these experiments were either derived from uni-specific cultures, or from mixed species occurring naturally in reservoirs. By applying the microscope-electrophoresis technique of charge measurement and making certain assumptions with regard to the geometry of the algae the electrokinetic potential and charge density of the algal surfaces were calculated. The theory and techniques employed by ABRAMSON *et al.* (1942) with proteins were extended and modified to be used for the algae.

Certain practical difficulties attended these experiments in that the *Bacillariophyceae* ('diatoms') tended to settle in the electrophoresis cell, and conversely certain *Myxophyceae* ('blue-greens') tended to rise. Filamentous algae also gave trouble because the migration velocity had to be observed strictly in a certain optical plane under the microscope. The filaments tended to trail through several planes making the observed velocity of doubtful value. These various difficulties were minimized by certain experimental techniques, and these methods, together with a more detailed description of the experiments, were given in a paper by the author presented to the Society for Water Treatment and Examination in 1956.

Charge Variation

The equation used to derive the charge density σ from the observed data is

$$\sigma = \frac{v\eta}{\chi}\left(\frac{4\pi Ne^2}{1{,}000DKT}\right)^{1/2}(\Sigma CZ^2)^{1/2}$$

where v is velocity of migration of an algal cell, χ is potential gradient, η is viscosity of disperse medium, N is Avogadro's number, K is Boltzmann's Constant, T is absolute temperature, D is dielectric constant, e is absolute electronic charge, and C and Z are the concentration and valency of any particular ion.

This indicates the dependence of σ (and similarly ζ, the zeta potential) on the ionic concentration in the water. With an ion-free distilled water the H^+ and OH' ions vary in concentration with pH, and cultures in such solutions show a marked correlation of σ with pH (Fig. 1) and ζ with pH (Fig. 2). In the natural (hard) reservoir water where many ions were present the effect of H^+ and OH' ion variation was not so marked. This was also shown when distilled water was brought to the same ionic concentration (ΣCZ^2) as reservoir water by adding a 0·0027M solution of magnesium sulphate. Interchange of cultured algae to reservoir water and natural algae to distilled water still showed the same result, indicating the effects were not due to the mode of growth of the algae.

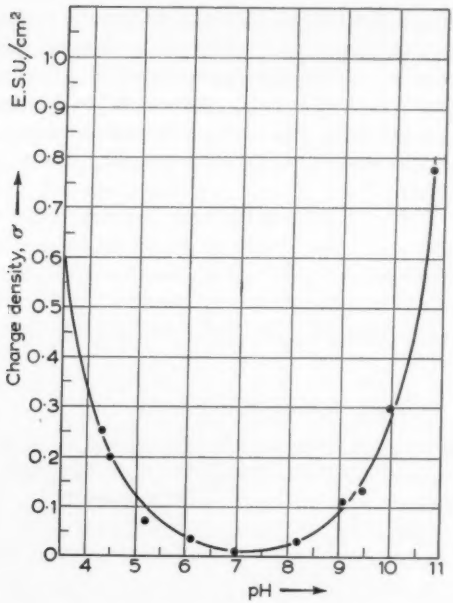

Fig. 1. *Chlorella*: Charge density—pH graph

However in all cases the algae remained electro-negative at all pH values investigated (approximately pH 2·5 to pH 11·5), i.e. no isoelectric point was exhibited. Minimum values of σ and ζ always appeared about pH 7–8.

At a constant pH it was found that the numerical values of zeta potential and charge density varied from genus to genus (and even between different species of the same genus). These facts have a practical significance as will be explained later.

Since in many practical situations algae are killed with chemicals before the engineer comes into contact with them, it was decided to investigate the effect of certain algicides on the algal charge. In practice where algae have to be controlled in large bodies of water, copper sulphate crystals ($CuSO_4 \cdot 5H_2O$) are added to the water in concentrations of up to 10 parts per million (p.p.m.). Experiments with two genera of naturally occurring diatoms, and

two artificially cultured algae, indicated that the copper sulphate increased the zeta potential slightly, and surface charge density more considerably. These tests were carried out at various pH values, ranging from 4·5 to 8·5. Although the numerical values for σ and ζ varied with pH, the percentage increase due to the copper sulphate seemed independent of pH. Extended contact

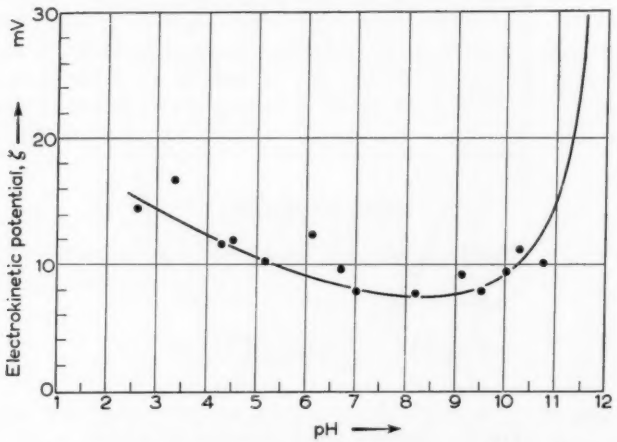

Fig. 2. *Chlorella*: Elektrokinetic potential—pH graph

between the copper sulphate and the algae (7 days) had little effect on σ and ζ. The algicidal chemicals iodine and formalin were also tested as they are sometimes used for laboratory killing of algae. Iodine at 2·5 p.p.m. showed similar effects to copper sulphate, with the exception that *Chlorella vulgaris* cultures at pH 8·1 showed very little charge variation. *Chlorella* at pH 5·0, however, did conform to the normal results. This anomalous behaviour of *Chlorella* is still unexplained. Formalin at 200 p.p.m. 600 p.p.m., 1,000 p.p.m. and 2,000 p.p.m. (normal laboratory doses) showed the reverse effect to copper sulphate and iodine, by reducing the values of σ and ζ. The amounts of the reductions appeared to be independent of pH and of the dose of formalin applied, within the range quoted above. Once again extended contact made very little difference. Finally, because of its

occasional use in the sterilization of water supplies, ozone was applied as an algicide. Here the doses applied were fixed by the ozone producing apparatus and were 1·6 per cent w/w and 2·7 per cent w/w. No extended contact could be tried with the ozone since it is not stable and rapidly disappeared in the water. In all cases the zeta potential and charge density were increased by the ozone, but no consistency could be determined with regard to the effect of the magnitude of the dose.

It has been suggested that as copper sulphate, iodine and ozone increase the electric charge and formalin reduces it, that the effects can be interpreted as oxidation-reduction potentials. As yet no satisfactory theory has been developed to meet this suggestion.

Theory of Removal

The experimental work briefly described above was undertaken to obtain information on the fundamental factors influencing the removal of planktonic algae from water. Obviously electrokinetic effects do not affect removal by mechanical means such as mesh filtration or centrifuging; but where surface adsorption effects are used the electrokinetic phenomena are significant.

Where chemical coagulants are added to the water to form an insoluble hydroxide precipitate which is flocculated, the object is to enmesh the algae in sufficient floc which, as it is sedimented, will carry down the algae with it leaving behind a clear supernatant water. This is the practice in certain waterworks installations treating lake or reservoir water for public supply.

A theory has been suggested that the removal of algae by flocs is due to the mechanical enmeshment of the algal cells. This explanation assumes that the water in passing through a floc blanket, or having floc particles falling through it, causes the suspended algae to be tangled in the floc, from which they are not freed. Arguments supporting this and dealing with the surface texture of the algae have been advanced by Carter (1948). Experiments by the author with flocs of different structure do not support this theory. It is not denied that such a mechanism might contribute to the removal, but it is suggested that it is not a primary cause, but only secondary. Another theory suggests that the flocs form and the algae are adsorbed on the outer

surface of the flocs, since these have a large specific surface. It is probably true that such a mechanism might be possible if the algae were added to the water after the floc was formed; but as in practice the suspended material is present before the chemicals are added this explanation is unlikely to operate in fact. Furthermore examination of flocculated material in the water always revealed the suspended impurity (algae or otherwise) well inside the floc particle. A protogel theory developed by HAY (1946), to explain the removal of all suspended materials in water, postulated the instantaneous formation of a fibrillar network of flocculent precipitates in which are water voids and the suspended impurities. This matrix breaks up with the impurities entrapped in the interstices of the flocs, and dewatering of the flocs takes place in which they shrink and densify in a process analogous to syneresis. The resultant flocs are dense, enmeshing all the impurities, and have clear water between them. If this theory is valid two observations should result from flocculation experiments. First, the appearance of the flocs should be instantaneous, and secondly, the flocs in their process of pseudosyneresis should appear to become progressively smaller. In fact neither of these results was observed, rather the reverse being true.

Knowing that normal water treatment hydroxides (ferric and aluminium) carry a positive charge, and having determined experimentally that the algae are negatively charged, the author believes that the basic mechanism is one of mutual attraction and charge neutralization of the algae and the incipient hydroxide flocculi. The consequences of this theory will be discussed below.

Adsorptive removal might also be employed by the filtration of water containing the algae through granular material without the addition of coagulants. The most common media thus employed is sand, although ballotini (uniform glass spheres) and crushed anthracite have also been suggested. Information regarding the charge carried by such granular sand filters is confused, and no information is available with regard to ballotini and anthracite. It is proposed to undertake an investigation on these lines and see if any valid theory can be developed.

Removal of the algae by a form of electrostatic precipitation has been mooted in casual discussion, but no serious attempt has been made to investigate the value of this suggestion.

Use of Flocculent Hydroxides to Remove Algae

The charge neutralization theory suggested above, to explain and predict the removal of algae by the use of flocculent hydroxides, has been tested by experiment. The theory would indicate that the algae present in the water would attract hydroxide precipitates to themselves and have an increasing floc building round these cells with time. This is shown in Fig. 3 where algae

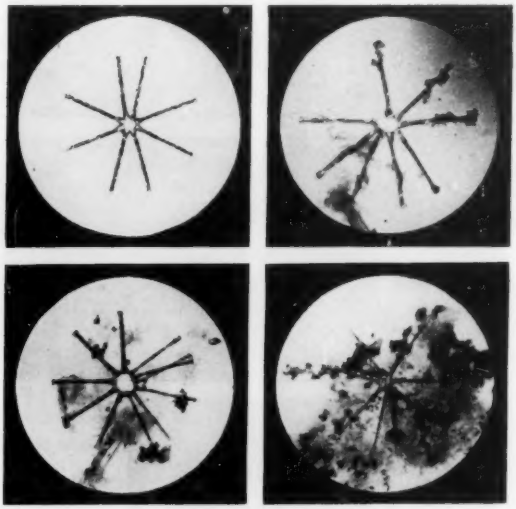

Fig. 3. Increasing floc formation on filaments of *Tribonema*

Time 0 min 0 sec. Time 0 min 20 sec.
Time 1 min 20 sec. Time 5 min 0 sec.

(*Tribonema*) taken from water with floc forming in it, at succeeding intervals of time, show an increasing growth of ferric hydroxide floc on the algae. Gentle stirring was taking place all the time, and the final stage shown occurs when the flocs became visible to the naked eye. A similar set of photomicrographs of *Asterionella* (a diatom) are shown in Fig. 4. The algae and flocs were stained with methylene blue for photographic purposes. Another result of the theory should be that floc formation should proceed more

rapidly in the presence of algae than in clear water. This is indicated in Fig. 5 where better floc formation is proceeding in jar B containing many million cells of *Tribonema* compared with

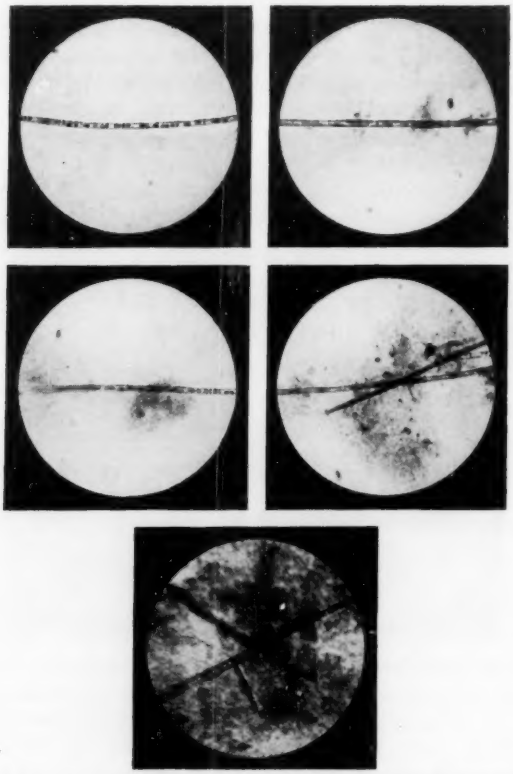

Fig. 4. *Asterionella* showing progressive accumulation of floc on frustules

jar A, containing clear water of identical chemical characteristics. Both jars were dosed simultaneously with 30 p.p.m. ferric chloride and had been stirring gently for the same time when the photograph was taken. It was also observed that visible flocs first appeared in jar B, and that when stirring was stopped the flocs in jar B settled more rapidly.

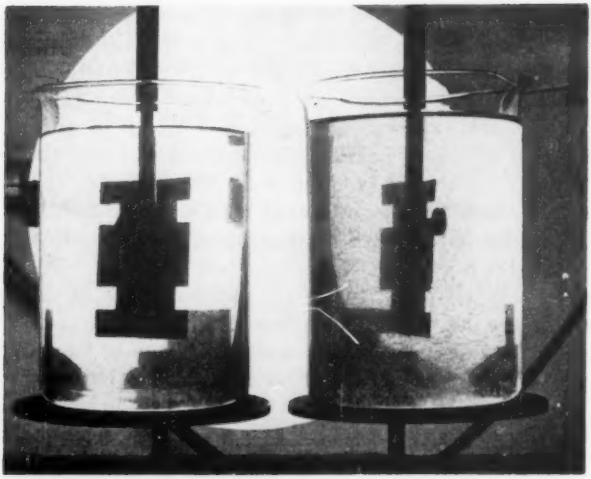

Fig. 5. Flocculation stirring test. Jar on left contains water free of algae but containing dissolved mineral salts. Jar on right contains same mineral salts, also many million cells of *Tribonema*. Both jars dosed 30 p.p.m. $FeCl_3$. Better flocculation is proceeding in jar with algae

Certain consequences arise from the theory and the experimental facts. These are:

(a) The charge density controls the quantity of floc required per unit area of algal surface.

(b) The higher the charge density the more floc will be required for charge neutralization.

(c) The larger the algae the more floc will be required for charge neutralization.

(d) The rate of migration of the coagulating colloidal precipitates will be (*inter alia*) a function of the algal zeta potential.

(e) To be most effective the hydroxide flocculi must be all electro-positive. (This is a function of pH.) (LARSON and BUSWELL, 1940.)

(f) Substances which affect the electrokinetic properties of algae (e.g. algicides) will also affect their floc carrying characteristics.

As the purpose of applying chemical coagulants to algae in water is to form enough floc round them to sediment them, it can be seen that these consequences are of operational significance. Tests with full-scale water clarifiers have shown (IVES, 1955) that the amount of chemical coagulant required to sediment an algal growth is largely governed by the number and size of the algae present. This is a function of consequences (a), (b) and (c). Item (e) has also been investigated with a water clarifier and it was found that better algal removal resulted from adjusting the pH with acid to a level which kept the (aluminium) hydroxide in a positively charged form. Item (f) explains some of the results reported in the literature for the flocculation of algae treated with copper sulphate and with chlorine, although it must be stated that some authors believe that better flocculation was induced in these cases by the surface roughening of the algae by the algicide giving a better 'bond' with the floc.

Although the engineering significance of these consequences has been discussed in relation to clarification of drinking water supplies, the same effects could be obtained if the removal of algae from sewage oxidation ponds was required, or their removal from industrial cooling water was necessary. However, if the algae were to be required for harvesting purposes for further treatment, chemical flocculation procedures would be unsuitable due to the difficulty of separating the algae and floc.

Separation of Algal Species

Independent work by KOLIN (1955) in Chicago indicated another application of the phenomenon of algal electric charge. Since different species of algae have different zeta potentials at the same pH, they will migrate at different speeds under the influence of an electric field. In his work Kolin took mixtures of *Ankistrodesmus* and *Chlorella* and caused them to migrate in a solution in an electric field to a boundary where there was a sharp increase in conductivity, hence drop in potential gradient. The speed of the migrating algae dropped sharply as they entered the region of a lower potential gradient so that they became concentrated in a narrow band. As this band progressed slowly through the solution, the algae of higher zeta potential migrated faster,

so that the band separated into two, the forward one slowly widening the gap between them. These bands formed what Kolin called mobility spectra. Using an overall potential gradient of only 5 V/cm at pH 7·3 he was able to separate the two algal species in 14 min. The sorting of different species of the same genus is almost certainly possible, and may even be extended to different varieties of the same species.

Acknowledgements

The experimental work described in the paper was carried out by the author in the Chadwick Public Health Engineering Laboratory of University College, London, and at the Kempton Park Experimental Plant of the Metropolitan Water Board, London.

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Production of L-Tryptophan in Submerged Culture

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Summary. A strain of *C. purpurea* has been isolated which will produce L-tryptophan from indole in submerged culture.
The L-tryptophan produced is extracellular and in a free form.
Levels of up to 1·5 mg/ml of L-tryptophan have been obtained.

Using mutant strains of *Neurospora crassa* the pathway anthranilic acid→indole(+serine)→tryptophan has been established by TATUM, BONNER and BEADLE.[6-8] Similar pathways have been reported to exist in *Salmonella typhosa, Escherichia coli*[1] and *Lactobacillus arabinosus.*[4]

We have isolated a strain of *Claviceps purpurea* (Fries) Tulasne which will produce uncombined, extracellular L-tryptophan from indole by fermentative means.

During the course of these investigations, TYLER and SCHWARTING[9] reported that washed mycelium of a strain of *C. purpurea* isolated from domestic barley ergot could utilize indole to produce tryptophan in replacement culture. Tryptophan disappeared from the replacement cultures in a relatively short time and nicotinic acid was identified as a product of tryptophan utilization.

Experimental Procedure

Strain. The strain of *C. purpurea* used in this study (hereafter referred to as strain C-1M) was isolated from a sclerotium of domestic rye ergot and maintained in pure culture.

Presented before the Division of Agricultural and Food Chemistry, Fermentation Subdivision, Symposium on Fermentation Process and Equipment Design, at the 132nd meeting of the American Chemical Society, New York, N.Y., 1957.

Media and fermentation. The general procedure for increasing the volume of inoculum and the details are as follows:

Parent or stock culture (slant)→slant→flask (100 ml)→ flask (1·0 l.)→fermentor (20 l.)

To initiate production of the inoculum, a part of the growth of a refrigerated culture of strain C-1M was transferred from the parent culture to a test tube slant. The culture medium was as follows:

	%		%
Glucose	2·0	$MgSO_4 \cdot 7H_2O$	0·025
Bacto-peptone	0·5	$FeSO_4 \cdot 7H_2O$	0·001
KH_2PO_4	0·05	Agar	2·0

pH not adjusted

After the slant had been incubated for 5–7 days at 26°C, a part of the vegetative growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of medium of the following composition:

	%		%
Glucose	1·0	Bacto-peptone	0·5
NaCl	0·5	Bacto-Beef extract	0·5
Sheffield NZA-amine A	0·5		

pH not adjusted

In some of the earlier studies, a synthetic medium was also used at this stage to better define growth and nutrition requirements of the organism. TABER and VINING[5] have reported the biotin requirement of three strains of *C. purpurea*. The composition of this synthetic medium is as follows:

	%		%
Glucose	2·0	$CaCl_2$	0·1
$(NH_4)_2SO_4$	0·2	$MgSO_4 \cdot 7H_2O$	0·05
L-Glycine	0·15	$FeSO_4 \cdot 7H_2O$	0·001
KH_2PO_4	0·27	Biotin	0·1 mcg/100ml

pH not adjusted

The flask was shaken on a reciprocal shaker at about 110 c/min with a stroke of about 3 in. The flask of culture was incubated with agitation at 26°C for 72 h, after which a 5 per cent inoculum was transferred to 1·0 l. of sterile medium in a 6·0 l. Erlenmeyer flask. This flask was equipped with a tube attached to a side vent

on the flask through which the contents of the flask were transferred to the fermentor. The composition of the medium is the same as the NZA-amine A-containing medium given above.

This flask was also aerated on the same reciprocal shaker. After aeration and incubation at 26°C for 48 h, this culture was used to inoculate the 40-l. stainless steel fermentor.

As none of the fermentation equipment is of unusual design, detailed descriptions are not given.

The fermentation medium (20 l.) was as follows:

	%		%
Glucose	2·0	KH_2PO_4	0·88
$(NH_4)_2SO_4$	0·4	$Na_2HPO_4 \cdot 12H_2O$	1·54
L-Glycine	0·15	Corn steep liquid (as received)	1·0

pH not adjusted

This medium was batch sterilized at 120°C for 10 min, cooled to 26°C, and inoculated with the 1·0 l. culture of strain C-1M. The fermentation was incubated at about 26°C with aeration by means of filter-sterilized air at the rate of 0·7 vol of air per vol of medium per min through a perforated stainless steel sparger. The agitation used was 400 r.p.m.

The indole solution was prepared by dissolving 2 g of indole in 200 ml of 25 per cent ethanol. This solution was kept in individual screwcap bottles at 37°C until time of addition.

Analytical Methods

Samples of whole broth were withdrawn aseptically. Aliquots of the filtrate were quantitatively assayed for indole by the method of WOOD, GUNSALUS and UMBREIT[10] and for L-tryptophan by the method of NASON, KAPLAN, and COLOWICK.[3] Quantitative determinations of L-tryptophan also were carried out directly on the filtrate (in the absence of indole) by a microbiological assay utilizing the organism *Lactobacillus arabinosus* 17–5. This assay was carried out essentially as described by GREENE and BLACK[2].

Paper partition chromatography was also used to qualitatively identify L-tryptophan. Portions of 0·1 ml of the filtrate samples were spotted on sheets of Whatman No. 1 filter paper and the chromatogram was developed overnight with 80 per cent ethanol

by the descending technique. Spraying with 1 per cent *p*-dimethylaminobenzaldehyde in 3·6 per cent HCl was used to detect the zones. With this reagent L-tryptophan gives a blue colour.

Crystalline L-tryptophan was obtained from the filtrate by an initial adsorption on Nuchar C-190-N carbon. A mixture (4 : 1 : 1) of *n*-butanol, 20 per cent ethanol, and distilled water at pH 9·6 was used to elute L-tryptophan from the carbon. The eluate was then concentrated to dryness, the solids dissolved in 80 per cent ethanol, and then concentrated to dryness again to yield the crystalline compound.

Qualitative identification of this compound as L-tryptophan was achieved by infra-red absorption and X-ray diffraction. The absorption curve as well as the diffraction pattern were identical with the L-tryptophan standard reference. In addition, the specific rotation of the compound using sodium light was $-35{\cdot}3°$ at 25°C at a concentration of 1 g in 100 ml of distilled water.

Results

Indole addition. Earlier investigations carried out in shake flasks indicated that indole could be added at various times during the fermentation. The results of some shake flask fermentations are given in Table I. Indole could not be added at the beginning of the fermentation without inhibiting growth and subsequent indole utilization. However, when indole was added at +24, +48, or +72 h, it was utilized with no apparent inhibition. It was also observed that maximum utilization was achieved when indole was added in a maximum quantity of 0·01 per cent.

In the fermentor, indole was added at a 0·01 per cent level starting at approximately +30 h and subsequently whenever the indole test was negative. Fig. 1 describes a typical fermentation in terms of indole addition. More than twice as much indole was added and utilized at the 0·01 per cent level than at the 0·002 per cent level even though the rate of addition was greater at the lower level.

Inconclusive data were obtained when serine was added with indole to the fermentation. No significant increase in indole utilization was noted. Paper partition chromatography of the

Table I. The effect of indole addition on indole utilization

C-1M (Synthetic medium, 100 ml/500 ml Erlenmeyer flask)										
Indole added 24 h	Indole remain. 48 h	Indole added 48 h	Indole remain. 72 h	Indole added 72 h	Indole remain. 96 h	Indole added 96 h	Total indole added	Indole remain. 7 days	mcg/ml L-trypto-phan bio-assay	mcg/ml L-trypto-phan colori-metric
5 mg	–	→					5 mg	–	42	52
5 mg	–	5 mg	–	5 mg	+		15 mg	+	91·5	112·5
		5 mg	+slight		+slight		5 mg	±	41	47·5
		5 mg	–	5 mg	+strong		10 mg	+	47	55·6
		10 mg	+slight		–	10 mg	20 mg	+	126	120

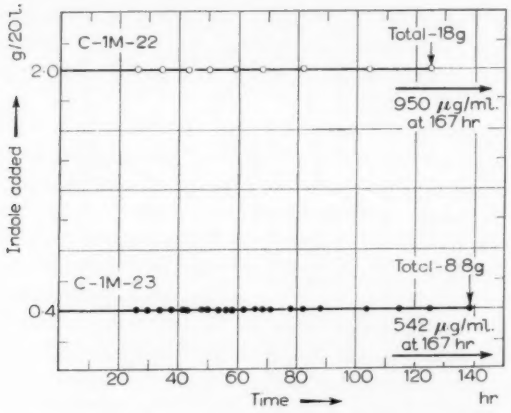

Fig. 1. The effect of indole concentration on indole utilization

fermentation filtrate showed only a slight concentration of serine. If serine is required for tryptophan formation from indole as in the *N. crassa* pathway, its formation by strain C-1M must be adequate.

Effect of pH *on indole utilization.* A marked dependence of indole utilization on pH was noted in preliminary experiments using shake flasks. When the pH was manually controlled, by adding 5N NaOH, at approximately pH 4, 5 or 7, it was observed that the optimum pH range for indole utilization was pH 5–6·5.

In Fig. 2 it is apparent that there is poor indole utilization above and below this pH range. In subsequent shake flask experiments and in the fermentor, the fermentation medium, as indicated above, was phosphate-buffered to prevent a rapid decrease in pH during the early stages of the fermentation.

L-*tryptophan production.* In the fermentor, indole addition was usually started at approximately + 30 h. Samples of the whole broth were withdrawn aseptically at various times and indole, L-tryptophan and pH determinations were carried out. The data for a typical fermentor fermentation are given in Fig. 3. It is interesting to note that the curve for rate of indole utilization parallels that for L-tryptophan production. An additional 2 per cent glucose was added at + 27 h since earlier observation (Fig. 4) had shown that there was little or no carbohydrate present in the

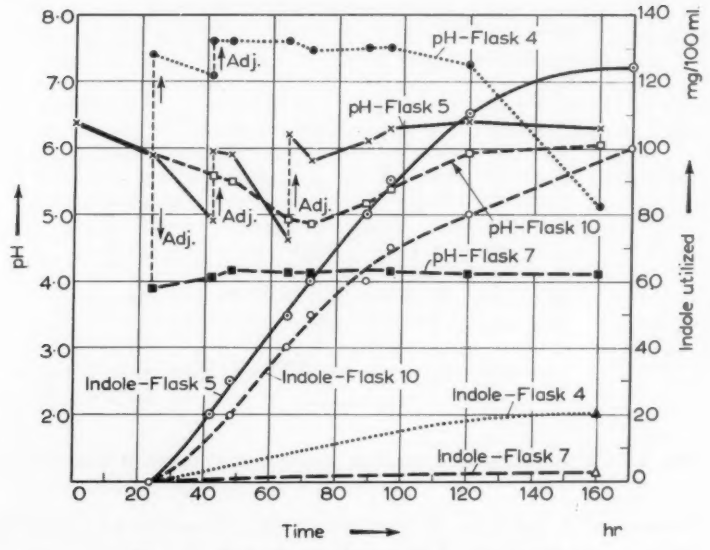

Fig. 2. The effect of pH on indole utilization

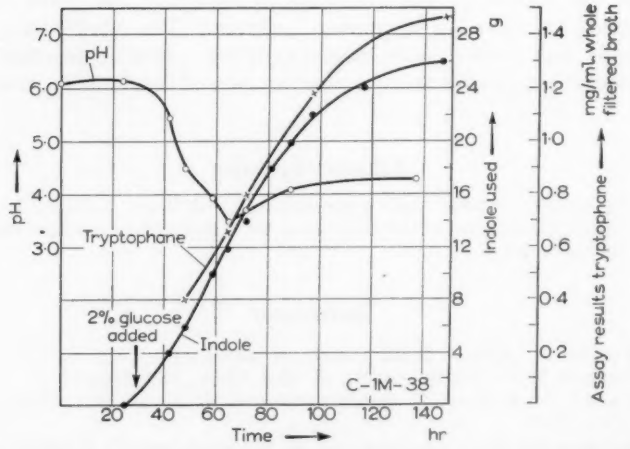

Fig. 3. Rate of formation of L-tryptophan following addition of indole

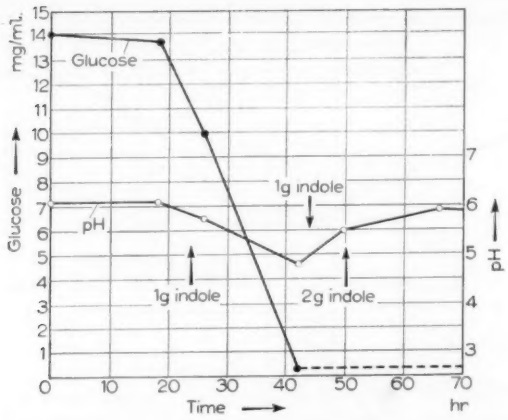

Fig. 4. Carbohydrate concentration during first 40 hours of fermentation

fermentation medium at this time. Simultaneous experiments carried out in shake flasks indicated that this supplementary addition of glucose was inhibitory to shake flask growth. However, the inhibition was overcome by buffering the shake flask contents at the time of glucose addition. The combination of glucose and buffer as a supplementary addition resulted in increased cell growth levels as well as rate of indole utilization.

Acknowledgement

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An Apparatus for the Culture of Algae under Controlled Conditions

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Summary. An apparatus is described in which large numbers of algal cultures can be maintained under the following conditions: (1) Constant temperature within the range 5 to 70°C, four different temperatures being maintained simultaneously; (2) uniform illumination at intensities up to 20,000 metre-candles; (3) continuous shaking at variable speed; and (4) aeration under aseptic conditions with mixtures of up to four different gases in any desired proportions.

Among other features, thermoregulator heads, flow-meters and heaters for sterile filters, of improved design are described.

To illustrate the use of the apparatus, data showing the effects of light intensity and temperature on the growth of *Monodus subterraneus* are presented.

Introduction

Various different types of equipment for large-scale and continuous culture of algae have now been described (MYERS and CLARK, 1944; BURLEW, 1953; KRAUSS and THOMAS, 1954; PHILLIPS and MYERS, 1954; TAMIYA, 1957; THACKER and BABCOCK, 1957). Apparatus for growing numbers of relatively small cultures under controlled conditions has received less attention (see FOGG, 1948; MYERS, 1950; ALGÉUS, 1951) but remains the most suitable for many kinds of physiological research. This paper is concerned with an apparatus of this last type; it is thought to be worth describing in some detail since it is intended for use in an extensive series of investigations and since it embodies several features which may be of interest to those constructing similar equipment.

In designing it, a main requirement to be met was that the apparatus should enable cultures to be grown simultaneously at several different accurately controlled temperatures anywhere

within the range in which algae grow in nature. It was also required that the illumination, which should be as uniform as possible over the area to be occupied by cultures, should be adjustable up to intensities fully saturating for photosynthesis. For maximum growth of unicellular algae a sufficient supply of carbon dioxide and agitation to maintain the cells in suspension are necessary. These conditions are best achieved by a combination of shaking and the passage of carbon dioxide-enriched air through the cultures (MILLER and FOGG, 1957). Finally it was required that the apparatus should accommodate culture vessels of a variety of shapes and sizes.

General Description

The main structure consists of four glass-bottomed thermostat tanks arranged linearly and supported on a 'Dexion' aluminium

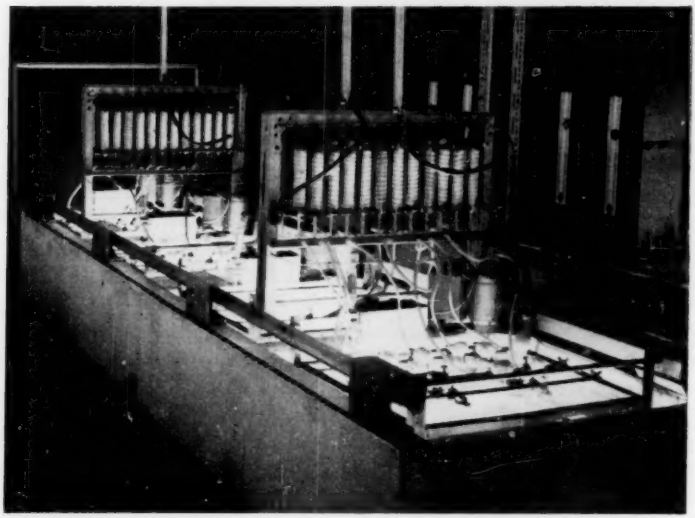

Fig. 1. General view of the culture apparatus. Tank No. 1 is nearest the camera and the housing containing the water tank and shaker motor furthest away. In the background on the right are the flow-meters and valves of the aeration system

alloy framework. Each tank is arranged to operate within a different temperature range, from up to 70°C for tank No. 1 down to 5°C for tank No. 4, which is refrigerated. Illumination is provided from below by banks of air-cooled fluorescent lamps. A frame, to which the culture vessels are clipped and which also carries the manifolds and sterile filters for aeration, is common to

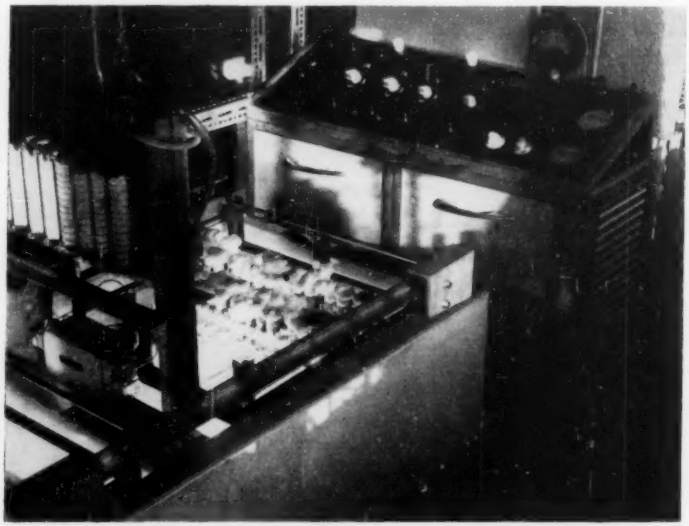

Fig. 2. Tank No. 1 and the control panel

all four tanks and can be oscillated in a direction parallel to the line of tanks by electrically driven cranks. Controls, pilot lights and fuses for the various electrical circuits are collected in a control unit at one end of the apparatus (Figs. 1 and 2). The mains electricity supply used is 230 V a.c., 50 cycles.

The Tanks

These are of mild steel, galvanized after all holes required for plumbing and fixing had been cut and, in the case of tank No. 4, the copper refrigerating coil had been 'sweated' on to the external

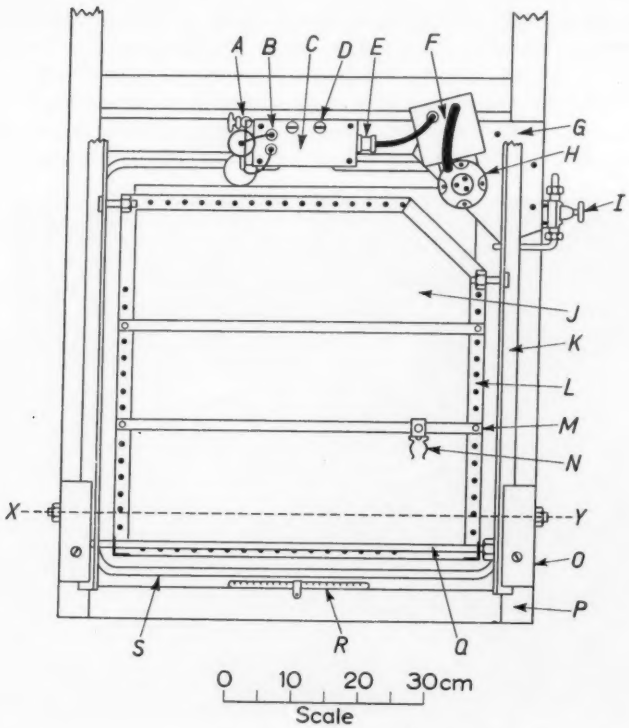

Fig. 3. Plan from above of tank No. 1. The finer details are not drawn to scale

A. Thermoregulator
B. Terminals for thermoregulator leads
C. Terminal box of heating element
D. Fuses for thermoregulator circuit
E. Mains supply plug for pump motor
F. Motor capacitor housing
G. Fixing plate for pump motor
H. Immersion pump
I. Diaphragm valve controlling water flow into tank
J. ¼ in. plate glass bottom of tank
K. Aluminium alloy T-section connecting shaking frames
L. Shaking frame of stainless steel angle
M. Stainless steel bars bolted to shaking frame
N. Sliding clips on bars of shaking frame
O. Bearing housing
P. Aluminium alloy strip on which bearing housings are mounted
Q. Stainless steel rod supporting shaking frame
R. Thermometer
S. Heating element
X–Y. Position of vertical section in Fig. 2

surface, and finally stove-enamelled white and given a protective coating of white anti-fouling paint. The top opening of each tank is 61 × 66 cm, the bottom opening, into which a panel of plate glass, $\frac{1}{4}$ in. (0·63 cm) thick, is sealed, is 51 × 56 cm and the volume of water normally contained is 90 l. (Figs. 3 and 4). It is essential

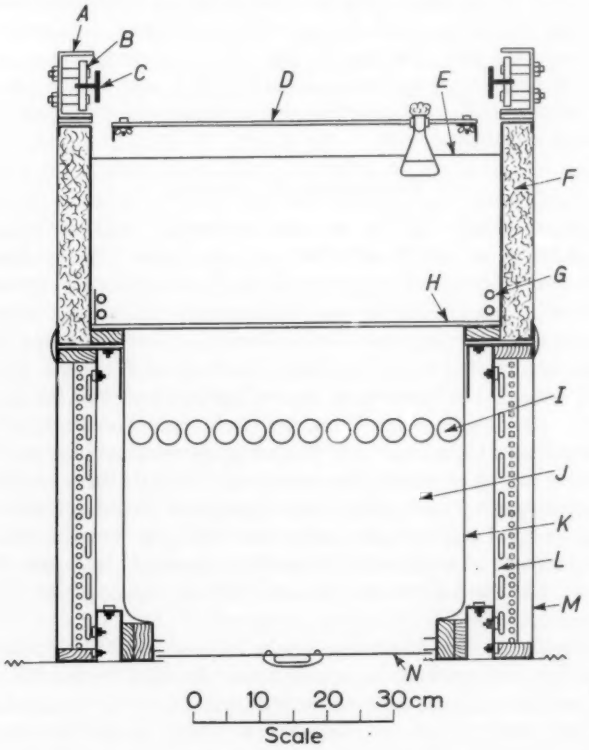

Fig. 4. Vertical section of tank No. 1 passing through the line XY in Fig. 1

A. Bearing housing
B. Ball-race
C. T-section connecting shaking frames
D. Stainless steel shaking frame
E. Water level
F. Cork insulation
G. Heating element
H. Plate glass bottom of tank
I. Bank of fluorescent tubes
J. Ventilating duct
K. Reflecting aluminium
L. 'Dexion' support
M. Hardboard panel
N. Sliding aluminium door

that the glass to metal seal should be water-tight; this was achieved by using a thick layer of 'Seelastic' mastic compound which was allowed three days to harden on exposed surfaces before water was slowly admitted to the tanks. This squeezed out excess mastic which was then trimmed off. For insulation, the sides of the tanks have a 5 cm layer of cork and bitumen between them and the sheet metal casing of the apparatus. In order that there may be a flow of water through them to make up evaporation losses, the tanks are interconnected through gate valves; in addition each tank has an overflow and a drain-valve leading into a common waste-pipe.

Temperature Control

Heating. Each tank has two electrical heating elements, formed to fit the walls with 0·6 cm clearance. These elements are of the conventional type, embedded in magnesium oxide and sheathed in nickel-plated copper tubing, and are of wattages chosen to give satisfactory control within different temperature ranges, *viz.* two of 2 kW for tank No. 1, two of 1·5 kW for tank No. 2, two of 1 kW for tank No. 3, and two of 500 W for tank No. 4. Electrical connections for each tank are made in a relatively water-tight box; one pair of leads are made common and the two heating elements are connected through three conductors in an insulated 7-core cable to a 3-position switch so as to give series, single and parallel element working conditions. The copper sheath of each cable is used as an earth, one pair of conductors for the stirrer motor and the remaining pair for the thermoregulator.

Cooling. A flow of cooling water for each tank is supplied via diaphragm valves from a constant head line fed from a 55 l. tank with a high-pressure ball-valve filling it from the mains supply. The refrigerator unit for tank No. 4, which is not shown in the figures, is a $\frac{1}{3}$ h.p. brushless motor driven compressor type. A double-pole change-over switch is provided in the refrigerator motor circuit giving connection to the relay for tank No. 4, for automatic control, or by-passing this for manual control. A 230 V a.c. solenoid-operated valve in the liquid line from the compressor has a similar switch giving either automatic or manual control.

Stirring. The water in each tank is stirred by an immersion pump chosen to give sufficient turbulence to ensure a minimum temperature lag and to damp waves produced by movement of the culture vessels in the water. Townson and Mercer miniature immersion pumps type 3 in stainless steel with $\frac{1}{30}$ h.p. brushless motors are used. Slight structural modifications to the pumps were necessary so that they fit into the corners of the tanks with a minimum obstruction of the illuminated area (Fig. 3).

Regulation. The control circuits are of the 'Sunvic' type. Tanks 2 and 3 are controlled by hot-wire vacuum switch units type F 103–4 (circuit 4). Tank No. 1 has a relay of the same type but the circuit is arranged so that in the 'high' and 'medium' positions of the switch only one element is controlled whilst in the 'low' position both elements are controlled. For tank No. 4 a hot-wire vacuum switch unit type F 202-10M (circuit 10) is used to control two-way load circuits with a single contact control device so that the refrigerator circuit is closed and the heater circuit simultaneously opened when the tank thermoregulator contacts close. The reverse occurs when the thermoregulator contacts open. A 'Metrosil' surge suppressor is included across the refrigerator circuit.

The precision thermoregulators are of the glass mercury-toluene expansion type. A feature of their design to which it seems worth while drawing attention is the use of Perspex (lucite) to make a completely insulated fine adjustment head (Fig. 5). A B.19 standard cone is turned out of stainless steel, to mate with the glass socket on the body of the regulator, and drilled to take a central brass rod with a tungsten needle fixed in it to form one contact. The needle carrier is spring-loaded and moves up and down without rotating as the adjustment screw is turned. The flexible insulated lead is connected to the needle carrier by a permanent soldered joint. This arrangement obviates the high resistance due to dirt and wear that is found with low current contacting devices.

Operation. Using the refrigeration for tank No. 4 and the cooling effect of water flowing from tank No. 4 into tank No. 3 and of tap-water flowing into tanks Nos. 1 and 2, it is possible to get precisely controlled temperatures ($\pm 0{\cdot}1$ °C) within the following ranges: No. 1, 20 to 70°C; No. 2, 20 to 50°C; No. 3, 10 to

35°C; No. 4, 5 to 30°C. A typical set of running conditions may be summarized as follows: tank No. 1 at 30°C with a flow of 20 l. per h of tap-water and heater switch set to medium (single heating element in circuit); tank No. 2 at 25°C with a flow of 15 l. per h of

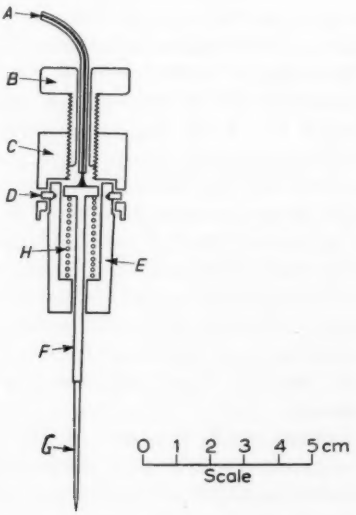

Fig. 5. Thermoregulator head

A. Insulated wire soldered to *F*
B. Perspex adjustment screw
C. Perspex body
D. Grub screw fixing *C* to *E*
E. Stainless steel standard B 19 joint
F. Brass disc and rod
G. Tungsten needle
H. Spring giving return movement to needle and disc assembly when unscrewing Perspex adjustment screw

tap-water and heater switch set to 'medium'; tank No. 3 at 20°C with a flow of water at 10°C from tank No. 4 and heater switch set to 'low' (heaters in series); tank No. 4 at 10°C with a flow of 10 l. per h of tap-water, refrigerator on automatic control and heater switch set to 'low'.

Lighting

Illumination is provided by two banks each of twelve 5-ft (152 cm) 80 W, white, reflector-backed 'Atlas' fluorescent tubes. These are mounted beneath the tanks, with the centres of the tubes 16·5 cm from the plate glass and spaced equidistantly, in a trough, 320 cm long, 71 cm wide and 46 cm deep, formed by the 'Dexion' angle framework supporting the tanks (Fig. 4). Externally the sides of this are filled in with hardboard, to cover the plumbing and wiring, and internally they are lined with reflector-

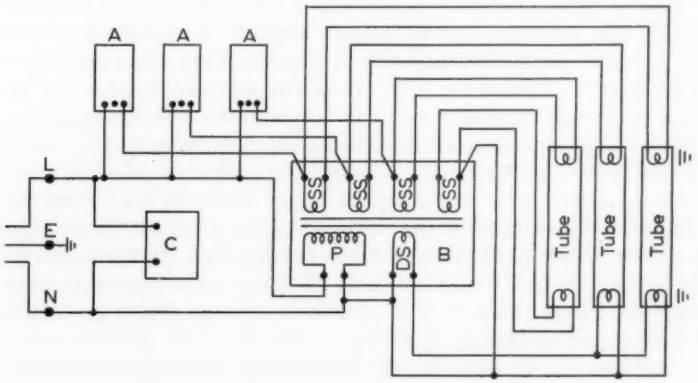

Fig. 6. Circuit diagram for lighting unit

A Choke BL/1090 (Atlas)
B. Quickstart transformer TR/2316 (Atlas)
P. Primary winding
DS. Double secondary
SS. Single secondary
C. Capacitor CRD/1138 (Atlas) 30 μf. 275 V.

grade aluminium sheeting. The bottom of the trough is formed by sliding aluminium doors giving access from below for servicing (the apparatus as a whole is at balcony level). At one end of the trough, under the shaking mechanism, is a 30 cm 'Ventaxia' fan which blows air around the lamps and out through an expanded aluminium ventilator at the other end.

A circuit diagram for the lighting unit is given in Fig. 6; the whole comprises eight such circuits which can be used in various

combinations to give light intensities at the level of the cultures from 5,000 to 20,000 metre-candles (m c). Tests with a photometer have shown that the light intensity does not vary by more than 5 per cent on either side of the mean value over the area occupied by the cultures with any combination of lamps. It is, of course, necessary to bear in mind the decrease in efficiency with age of fluorescent lamps and to check light intensities for every experiment.

The Shaking Mechanism

Two lengths of T-section aluminium alloy running in bearings along the sides of the apparatus support a square frame of 53 cm side, made of stainless steel angle, in each tank parallel to and about 1·5 cm away from the water surface (Figs. 3 and 4). One corner of each frame is cut to clear the immersion pump. Each frame is drilled so that stainless steel bars may be fixed across it in various positions by means of bolts and wing nuts. The culture vessels are fixed to these bars, so that they are sufficiently immersed in the water bath, by means of Terry clips bolted to slightly modified rubber tubing clips (Baird and Tatlock, No. B21/007) which clamp on the bars. This system enables culture vessels of up to 1 l. capacity to be held in any desired position on the shaking frames.

The aluminium alloy angles which carry the frames each run in four sets of bearings so placed as to give maximum support and rigidity to the assembly. Each bearing unit is made of an aluminium alloy channel section holding three ball-bearings; two on either side of the horizontal web of the T-section to hold it in the vertical plane and one running against the edge of the horizontal web to align it horizontally. The aluminium alloy angles carry at one end a stainless steel rod, mounted in ball-races, passing through the journals of connecting rods of a pair of cranks with a 3·2 cm throw. These cranks are driven through a five-step pulley connected by a belt to a matching pulley on a counter shaft to give five steps in speed on the final drive shaft. The counter shaft is driven by a 30·5 cm diameter pulley connected to a 4·5 cm diameter pulley on the motor shaft. The motor is a $\frac{1}{4}$ h.p. Hoover capacitor induction type, the speed of which can be varied slightly through a 'Variac' transformer in the control

panel. With the motor running at 1,425 r.p.m. the speeds obtainable on the final drive shaft are 66, 88, 120, 152 and 184 r.p.m. An electrical tachometer is connected to the latter to indicate shaking speed in oscillations per min on the control panel.

The Aeration System

For most purposes it is sufficient to aerate cultures with air enriched with carbon dioxide. A suitable mixture is provided for the apparatus by combining air from a diaphragm pump with medical quality carbon dioxide from a cylinder. The two gases are taken via constant pressure tanks and passed after humidification through Quickfit flow-meters of the vertical type before being mixed. By means of interchangeable glass jets in the flow-meters, different proportions of carbon dioxide in air may be obtained. The mixture is then distributed through a T-junction, on either arm of which is a Saunders diaphragm valve, to the two groups of manifolds.

A second system enables mixtures of up to four different gases to be used (nitrogen, oxygen, argon and carbon dioxide are being used in current experiments with nitrogen-fixing algae). With the exception of carbon dioxide, which is taken via a constant pressure tank, the gases are supplied from commercial cylinders through reducing regulators of the British Oxygen Company 'Endurance' M.S.51 type. The flow of each gas is measured by glass flow-meters of original design (Fig. 7) having mercury as the manometer fluid. By means of interchangeable internal jets of varying sizes the proportion of the gases may be varied within a wide range. After each flow-meter a fine control needle valve enables small adjustments to be made and 'blow-back' on shutting down to be prevented. After being mixed the gases are humidified by passage through distilled water in a 4-l. gas washing bottle fitted with a sintered glass distributor. The mixture is distributed in the same way as in the other system.

From the diaphragm valves the gas mixture passes to a manifold which distributes it via small needle valves and sterile filters to the cultures. The manifolds and filters are mounted on the shaking frame (Figs. 1 and 2) in two groups of 24, one serving tanks Nos. 1 and 2 and the other tanks Nos. 3 and 4. The filters

(Fig. 8) consist of cotton-wool filled glass tubes which, being connected to them by silicone tubing, can be sterilized together with the culture vessels. Each filter is contained in a heater jacket so that it can be maintained a few degrees above room temperature to prevent condensation in it and consequent con-

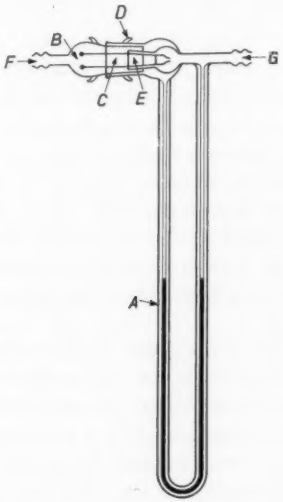

Fig. 7. Flow-meter

A. 1 mm bore capillary tube
B. Standard B 24 joint
C. Flow-meter jet
D. Hook for spring
E. Standard B 10 joint
F. Gas inlet
G. Gas outlet

tamination of the culture. The jackets consist of pieces of aluminium alloy tubing, 15 cm long and 2·5 cm diameter, covered with a double layer of glass cloth and wound with nichrome wire of a suitable gauge to give a resistance of about 6 ohms. The wire is connected with terminals on bands of pure aluminium 1 cm wide at either end of the tube, which clamp the glass cloth in position. Terry clips are arranged to hold the jacket and make electrical contact through the bands to a positive bar mounted on insulating material and the metal frame which acts as the negative

earth connection. The heaters are thus connected in parallel. Braided copper wire strips lead to overhead bus-bars of pure aluminium carrying the current. To ensure safety with a minimum of insulation the current is of low voltage, being supplied from a transformer giving 10 V a.c. at 80 amps.

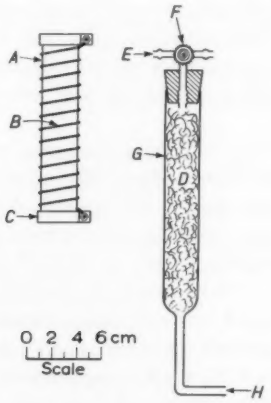

Fig. 8. Sterile filter and heating jacket

A. 1 in. (I.D.) aluminium alloy tube covered with glass cloth
B. Nichrome wire winding
C. Pure aluminium band clamped by nut and bolt
D. Cotton wool
E. Gas inlet
F. Tap with knurled adjustment screw
G. Glass tube
H. Gas outlet

The Growth Characteristics of *Monodus subterraneus*

Using this apparatus, a study has been made of the growth and cellular characteristics of the unicellular xanthophycean alga *Monodus subterraneus* Petersen at various temperatures and light intensities. In previous work (MILLER and FOGG, 1957, 1958; FOGG and MILLER, 1958) this organism was cultivated at 25°C and at a light intensity of about 3,600 m c, but although there were indications that relative growth rate was not greatly increased by employing a higher light intensity no data existed concerning the optimal temperature for growth.

The alga was grown under aseptic conditions in modified conical flasks of standard mineral medium (MILLER and FOGG, 1957), continuously shaken and aerated with 1 per cent carbon dioxide in air. The light intensity on the vessels was approximately 15,000 m c, while an intensity of about one-quarter of this value (3,700 m c) was obtained by placing screens consisting of two thicknesses of black nylon fabric around the bottoms of certain of the culture flasks. The lower intensity was selected to approximate to that used in the earlier growth experiments. Duplicate cultures were employed for each set of physical conditions studied.

In order to observe growth over the range 10–35°C at 5° intervals two experiments were performed, control cultures at 25°C being employed in each case. The inoculum for each experiment was taken from a 12-day-old stagnant culture grown at laboratory temperature (22–25°C), and gave an initial cell concentration of about 100 cells/mm^3. As in previous experiments employing the above culture conditions, growth was estimated: (a) by calculation of the relative growth constant, k, in $\log_{10}$ units/day from a number of cell counts with a haemacytometer during exponential growth; (b) by determination of final cell concentration; and (c) by determination of final dry weight/unit volume of culture.

The results of these experiments are presented in Table I. No growth occurred at 10 or at 35°, although cultures taken from the 10° tank after 13 days and from the 35° tank after 10 days proved to be viable on transferring to the 25° tank. At 15° there was an appreciable lag phase at both light intensities before exponential growth began. Organisms that had been adapted to this temperature through two serial subcultures in the 15° tank showed little increase in k, although the lag phase on subculturing was eliminated.

The table illustrates the following points:

(1) The optimal temperature, i.e. that allowing the most rapid and abundant growth, is 25°C. Only at this temperature was a statistically significant difference found between k values at low and high light. (2) The value of k at 25° and 15,000 m c (corresponding to a mean generation time of 24·3 h) represents an increase of only 19 per cent for a fourfold increase in light intensity. Thus the growth of *M. subterraneus* is not seriously light-

Table I. Growth and cellular characteristics of *Monodus subterraneus* at different temperatures and light intensities

	Low light (3,700 m c)				High light (15,000 m c)			
	15°	20°	25°	30°	15°	20°	25°	30°
k $\log_{10}$ units/day	0·081	0·177	0·250	0·163	0·074	0·191	0·297	0·169
Standard deviation of k	0·0037	0·0043	0·0071	0·0055	0·0052	0·0071	0·0181	0·0080
Mean final cell conc. (10^3 cells/mm^3)	237	465	552	101	283	513	718	257
Mean final dry wt. (mg/10 ml culture)	64·5	72·9	82·8	41·0	74·1	94·5	122·0	72·1
Relative dry wt. (mg/10^7 cells)*	0·27	0·16	0·15	0·41	0·26	0·18	0·17	0·28
Packed cell vol. (mm^3/10^7 cells)*	—	—	0·44	—	—	—	0·44	—
Chlorophyll *a*/cell (arbitrary units)*	—	—	2·9	—	—	—	1·0	—

* In the stationary phase.

limited at the intensity of ~ 3,700 m c employed here and in earlier experiments. (3) Relative dry weight of cells at the cessation of growth was found to vary according to the physical conditions. At both light intensities it was at a minimum at 25°C, while varying over a much wider range at low than at high light. Many giant cells, almost spherical in shape and up to 10·5 μ in diameter, were observed in the 30° cultures at all stages of growth. The association of low relative dry weight with optimal growth conditions has been noted previously (Miller and Fogg, 1957).

Considerable differences in pigmentation occurred between cultures grown in low and high light. The pigments of *Monodus* are extremely difficult to extract in the cold, and only the following technique has been found satisfactory. Cells are centrifuged,

transferred to an agate mortar with acetone and ground vigorously with glass powder for at least 5 min. The ground material is then extracted several times with cold 95 per cent methanol, and the optical density of the solution of chlorophyll *a* so obtained is determined at 660 mμ. A comparison of the chlorophyll *a* content per cell grown in low and high light at 25°C is given in the table. Parallel determinations of packed cell volume were made, cells being centrifuged in haemocrit tubes for 45 min at 1,750 g. No significant difference in packed cell volume of low-light and high-light cells was found.

Discussion

In broad outline the results for growth at different temperatures resemble those obtained for various chlorophycean algae. RYTHER (1954) obtained no growth of the marine algae *Nannochloris atomus* and *Stichococcus ?cylindricus* at 10°, though both grew rapidly over the range 15–30° with no clearly defined optimum. Growth rates decreased considerably at 30°. The optimal temperature for *Chlorella pyrenoidosa* is stated to be 25° (DAVIS *et al.*, 1953), while GEOGHEGAN (1953) found this temperature to be optimal for *C. vulgaris* growing in mineral medium, only poor growth occurring at 20 or 30°. SPOEHR and MILNER (1949), however, showed that the highest yields of *C. pyrenoidosa* occurred at different temperatures according to the composition of the medium and the light intensity. This effect of the composition of the medium may explain the results of NEISH (1951) who stated that the optimal temperature for *C. vulgaris* growing in glucose medium is 35°. In the case of *Monodus* there appears to be a pronounced optimum at 25°C for all aspects of growth, at both light intensities employed. although the effect of varying the composition of the medium is not known.

It is generally agreed that the chlorophyll content of algal cells is lower, the higher the light intensity at which they are grown. TAMIYA *et al.* (1953) observed that *C. ellipsoidea* cells are a paler green when grown in intense light, and that this effect was also dependent on temperature: at higher temperature a higher light intensity was required to produce pale cells. SARGENT (1940) found that *C. pyrenoidosa* cells cultivated under intense illumination had a chlorophyll content of approximately one-half that of

cells cultivated at one-seventh of the intensity, yet had a higher capacity for photosynthesis. On the other hand MYERS (1946) found with *C. pyrenoidosa* grown in continuous culture that there is a concomitant variation in cell number and chlorophyll per unit volume of cells. With increasing light the cells become increasingly large but with about the same chlorophyll content per cell. The highest intensity he employed was only 3,875 m c (incandescent light). The results obtained with *Monodus* show that its chlorophyll content per cell is much lower at 15,000 m c than at 3,700 m c (fluorescent light), in common with most of the findings on *Chlorella*, and that there is no difference in size of cells at the two intensities employed, at least when growth is complete.

Acknowledgements

Two of the authors (G.E.F. and J.D.A.M.) wish to record their appreciation of the work of their colleague (W.E.E.S.), to whom the credit for the design and construction of the apparatus is largely due.

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The Separation of Flour into Fractions of Different Protein Contents by Means of Air Classification

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Summary. Fractions of markedly different protein contents may be obtained by air-classifying flour (in the sub-sieve range) at cut sizes of about 17 and 35 μ respectively. Of these fractions, the fine, 1 (of high protein content), contains fragments of interstitial endosperm protein and small starch granules; the intermediate, 2 (low protein), mainly large starch granules; the coarse, 3, fragments of endosperm cells. The yields and protein contents of the fractions vary with different wheats; the yields of 1 and 2 may be greatly increased through suitably grinding the flour after milling.

In general, 1 may be used for raising the protein content of flours deficient in protein to a level suitable for bread-making. The amount of 1 required depends on its protein content and on whether it came from a hard or soft wheat, the hard wheat fraction having better bread-making qualities. The resulting variation in the proportion of 1 required in the blend causes variation in the proportion of other particulate materials introduced with 1, in which they have become concentrated during the air classification. These include diastatically active agents and finely divided discolouring matter; the level of the former reached in the blend greatly affects its bread-making value. Pre-grinding of the flour may be helpful in this respect.

When chlorinated, 2 is potentially useful for making light-structured cakes, but hard English wheats have mainly given less satisfactory results than soft. Under certain conditions, 3, with or without 2, may be better for biscuit-making than the parent flour.

Introduction

In Great Britain about 60 per cent of the flour milled is used for bread-making, the remainder being used for making cakes and biscuits and other edible commodities. While for bread-making, the protein content of the flour must be at a sufficiently high level,

preferably over 11 per cent, for certain cakes it should not exceed 6–7 per cent.

Wheats of high protein content, and certain wheats of low protein content which are particularly suitable for cake-making, must be imported, and are relatively expensive. Flour from home-grown wheat tends to have an intermediate protein content, often say 9–10 per cent. If it could be separated into different portions, of high and low protein contents respectively, the requirements of the different consumers might be better met. This is the practical background to the work described in the following paper.

The Particulate Nature of Flour

In flour milling, particles of wheaten endosperm are reduced in size gradually by means of smooth rolls in order that the flattened bran and germ fragments may pass over a sieve through which the flour produced by the reduction is able to fall. The aperture size of the flour sieve varies, according to the stage and conditions of milling, over a relatively small range: roughly from about 100 to 150 μ* (the approximate aperture lengths of Nos. 14x and 9xx Swiss bolting silks, respectively).

This range of sizes is of the same order as the dimensions of the cells of which the endosperm of the wheat grain is composed. In the milling of hard wheats a large proportion of the endosperm cells escape disruption but become separated or form part of small conglomerates of cells or segments of cells. In this condition they may pass through the silk to form part of the finished flour.[4, 9] Some of the cells (the proportion being large with soft wheats) break up during milling into particles ranging from under 1 to about 100 μ in length or diameter.

The cells contain starch granules, which, broadly, fall into two groups: (1) spherical or slightly polyhedral granules ranging in diameter from under 1 to about 10 μ; (2) lenticular granules, from roughly 15 to over 40 μ. The starch granules are embedded in a matrix of protein, the whole being enclosed by the thin wall of the endosperm cell. When the cells break, starch granules belonging

* Exceptionally fine silks with an aperture size of about 65 μ have been used in preparing flours for particular purposes, e.g. the manufacture of special cake flours.

to the second group become detached relatively easily from the protein matrix in which the small starch granules are more firmly embedded. A diagrammatic representation of the process is given in Fig. 1, the upper part of which shows two types of endosperm cell present in different parts of the wheat grain. The matrix itself breaks up during milling into particles of various sizes, including fragments of free protein. These were, from their appearance, described as wedge-protein by HESS in his classical work on the form of occurrence of protein in endosperm.[7a, b]

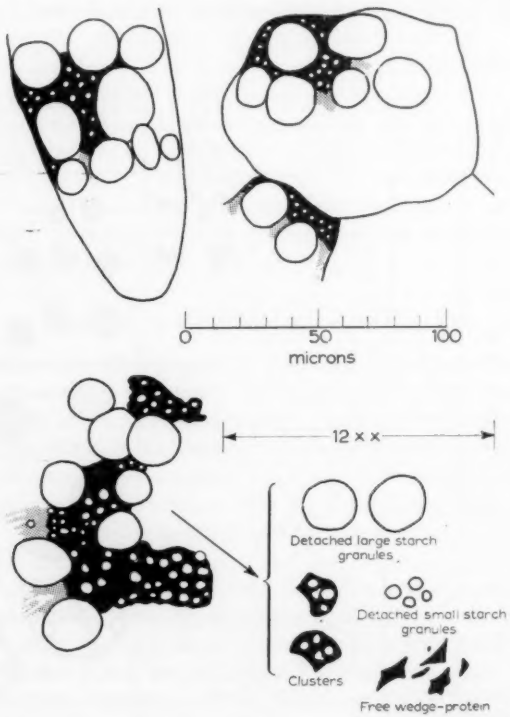

Fig. 1. Upper part. The two main types of endosperm cell: prismatic (*left*), polyhedral (*right*). Lower part: Exposed cell contents and products of further breakdown (12 xx is the representation to scale of the mesh aperture of a typical flour bolting silk).

Size-grouping of Flour Particles

It therefore happens that the particles present in flour milled from soft wheat may be grouped by size as in Fig. 2. Those shown in the upper part of column 6 may be described as 'clusters' for want of a more convenient term; they represent fragments of the protein matrix in which small starch granules are embedded; above 28 μ fragments of endosperm cell may be recognized.

Thus, the particles up to about 17 μ include pieces of free protein, small 'clusters' and detached small starch granules. In this fraction the proportion of total protein to starch (and hence the

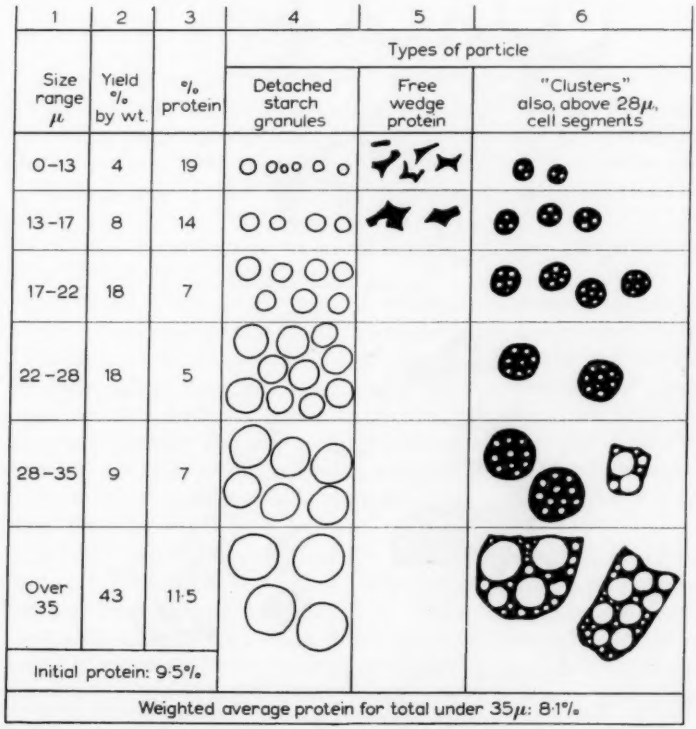

Fig. 2. Proportions and nature of particles of various sizes present in a flour commercially milled from English wheat

protein content of the fraction) is much higher than in the total flour. The next (intermediate) fraction—say 17–35 μ—contains a large proportion of free starch granules, and is therefore relatively low in protein content.

Practical Possibilities of Separation

Practical schemes for 'protein shifting' on the above basis involve classification in the sub-sieve range, because ordinary sieving processes do not afford a 'cut' below about 40 μ (Fig. 2 shows that the protein differentiation produced by a cut at 35 μ was limited to 3·4 per cent). Apparatus in which the effect of air-drag on individual particles is opposed to that of centrifugal force has been developed during recent years for sub-sieve classification of powders. Two such apparatuses which we have investigated closely are known as the Bahco Elutriator and the Mikroplex Spiral Air Classifier. The latter machine has been employed by HANSSEN and NIEMANN,[6] who reported in a pioneer paper that a fine fraction of relatively high protein content could be obtained from flour by means of it.

The Bahco and Mikroplex Air Classifiers

The Bahco classifier is a laboratory batch analyser for handling only about an ounce of material, manufactured (under licence from B. A. Hjorth & Co.) by Établissements Neu of Lille, France. It has been described by LIPSCOMB.[10] The Mikroplex is a production machine for continuous operation, made (by Alpine A.G., Augsburg, Germany) in three sizes with capacities ranging from about 1 cwt to over 1 ton per hour; it has been described by RUMPF and KAISER.[11]

Both machines make a cut into two (fine and coarse) fractions in one operation. The cut size may be readily varied by means of adjustments affecting the air flow, but its actual value in terms of particle size must be determined by microscopical measurement on the fine fraction. Measurement of upper particle size is, naturally, relatively simple in the case of closely sized fractions. In flour-milling practice, the adjustment of the Mikroplex could be controlled in the first place by observation of the weights of separations made and, ultimately, by protein determinations on

the products. With either machine, Bahco or Mikroplex, the coarse fraction may be re-fed and progressive cuts made, commencing (normally) by removing the finest material.

In general, we have found good agreement between results given by the two machines on percentage yields and on protein contents of all fractions except the finest. The fine fraction separated by the Bahco is generally up to 3 per cent lower in protein content, and it may in some cases be slightly lower in amount, than that separated by the Mikroplex.

In explanation, the Bahco is constructed to trap all except the very finest material, which is allowed to escape to atmosphere. The weight of the finest material is meant to be obtained by difference. This method of operation works satisfactorily with dry inorganic powders for which the apparatus was designed, but with materials such as flour, that behave hygroscopically, it is quite difficult to ascertain the exact amount of this small loss; the fine fractions are so small in amount that though they can be weighed they cannot be tested for moisture content by ordinary methods. Evidently, a small proportion of the flour exists in the form of free protein particles so minute that they escape being trapped by the Bahco fine catchment trough.

The Bahco analyser was found suitable for use on flour, with the following provisos: (1) the vibratory feeder required partial reconstruction to intensify the vibration; (2) in order to obtain satisfactory feeding and dispersion of specially fine or soft flours it was found necessary to pre-mix them with a proportion ($\frac{1}{3}$ or $\frac{1}{2}$) of granular flour or middlings milled from hard wheat. Check tests showed negligible break-down when the coarse additive was fed alone. The weight of the additive was deducted from that of the coarsest separation before calculating the percentage analysis of the sample under examination. Check trials on lively flours, made with and without the additive, gave similar results.

Our experiments with the model No. 132 MP. Mikroplex have shown that the proportion and protein content of the fine fraction separated, at a nominally constant cut-size regulation, are affected by variation in rate of feed, especially at high feed rates. In comparative tests on different flours, therefore, the rate of feed has been kept more or less at a point (1 cwt per hour) about mid-way in the useful range afforded by the machine.

Performance of the No. 132 MP. Mikroplex, at Various Adjustments, on an English flour

Table I and Fig. 3 show results of tests on an English flour of 9·3 per cent protein content and 13·7 per cent moisture content. The operation was progressive, the coarse fraction at each stage (reading from top to bottom of Table I) forming the feed to the next stage with an appropriate increase in the cut-size regulation. The protein contents of the successive fine fractions ranged from 22·7 to 4·2 per cent. Fig. 3 shows the corresponding values for the accumulated fines, and can be used to calculate approximate values for fractions covering various ranges of particle size. It

Table I. Data on various fine fractions obtained from English flour (of 9·3 per cent protein content*) by means of progressive, or multi-stage, operation of the Mikroplex classifier, the coarse fraction at each stage being refed.

Setting of cut-size regulator (°)	Successive fines		
	Maximum particle size (μ)	Yield (% of initial feed)	Protein content (%)
5	5·5	1·3	22·7
10	7	1·9	19·7
15	9	1·5	17·8
20	11	2·2	16·6
25	14	2·9	13·1
30	18	2·0	10·4
35	21	5·9	6·0
40	24	9·7	4·2
45	28	13·6	5·2
50	33	19·0	8·6

* Protein contents are expressed on a 14% moisture basis.

also serves to show how the values for upper particle size in the fines respond satisfactorily to changes in the cut-size regulation, which are shown on the horizontal scale of the graph in terms of the Mikroplex scale degrees (arbitrary units). For any given regulator setting, the corresponding yield, protein content and maximum particle size of the fines are given by following a vertical line on the graph to its intersections with the respective curves and then reading horizontally to the vertical scale, which is common to

the three sets of values. Thus, at a setting of 47°, the yield of fines was 48 per cent, with a protein content of 8 per cent and a size limit of 30 μ. Fig. 3 shows an accelerated increase in yield and fall in protein content as the size limit of the fines begins to exceed 19 μ, i.e. enters the range in which large starch granules are abundant. Later, from 28 to 40 μ, the protein content of the

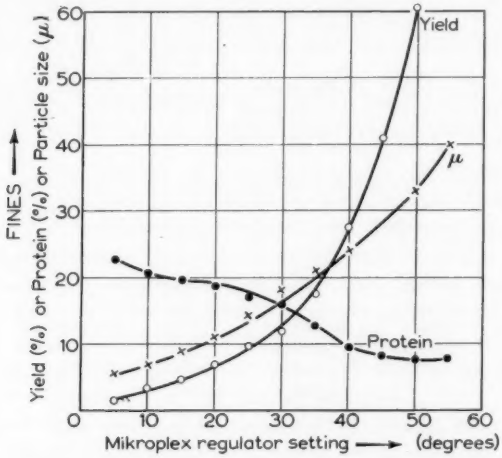

Fig. 3. Variations of yield, protein content, and maximum particle size of fine fractions (accumulated fines calculated from values for successive increments shown in Table I) with progressively coarser settings of cut-size regulator of Mikroplex No. 132 MP

accumulated fines is almost steady, at about 1 per cent below that of the initial flour, although (from Table I) the successive increments of fines rise sharply in protein content above 24–28 μ; this rise is due to the entry of fragments of endosperm cells which accompany the detached larger starch granules. In general, to obtain maximum yields of fractions which are reasonably high and low, respectively, in protein content, the most appropriate cut-sizes are about 17 and 35 μ. In certain directions, however, the effects of a shift in these cuts of only 2 or 3 μ are quite marked. Table II shows, for example, the effects of maintaining the coarser cut at 35 μ while increasing the size limit of the fines; the values

given were obtained by calculations based on Fig. 3. As would be expected, the yield and protein content of the fines are markedly affected; on the other hand, the intermediate fraction is affected only slightly in protein content over a comparatively wide range of variation in yield. The intermediate fraction, however, is affected markedly, both in yield and protein content, by relatively small changes in the coarser cut. The yield and protein content of the coarse fraction are also markedly affected by these changes.

It is evident from Table II that with this soft English flour two

Table II. Effects of changes in cut-sizes on yields and protein contents* of the three fractions from soft English flour of 9·3 per cent protein content

Cut Sizes		Fraction					
		Fine		Intermediate		Coarse	
Fine μ	Coarse μ	Yield %	Protein %	Yield %	Protein %	Yield %	Protein %
A. Coarse cut held at 35 μ; *fine cut varied*							
13	35	8·5	18·0	56·5	6·5	35	11·7
15	,,	10	17·2	55	6·2	,,	,,
17	,,	12	16·2	53	6·1	,,	,,
20	,,	17	13·2	48	6·2	,,	,,
23	,,	24	10·2	41	6·7	,,	,,
B. Fine cut held at 17 μ; *coarse cut varied*							
17	35	12	16·2	53	6·1	35	11·7
,,	30	,,	,,	36	5·3	52	10·5
,,	27	,,	,,	25	5·25	63	9·6

* Expressed on 14 per cent moisture basis.

classifications (by means of two machines such as the Mikroplex, working in sequence) would provide three fractions, of which the extremes in particle size could be blended, the final result being two products: (1) half the initial flour with a protein content 3 per cent higher; and (2) half with a protein content 3 per cent lower than the initial value. Alternatively, one classification at a cut-size of 17 μ would give 12 per cent of the original flour at a protein content raised to 16 per cent, the balance of the flour having been reduced in protein content from 9·5 to 8·7 per cent.

The levels of yield and protein content of the three fractions, however, are markedly affected by both type and protein content of the wheat from which the flour has been milled. Table III shows the values of fractions from series of flours, laboratory milled to about 70 per cent extraction, from ranges respectively of soft and hard English wheats, mostly of specified strains. The protein contents of the fine fractions from soft wheat flours tend to be

Table III. Proportion and protein contents* of the three main fractions constituting flours Bühler-milled from various soft and hard English wheats

Soft Wheats

Variety	Hybrid 46	Yga	Yga	Cappelle	Minister	Mixed unspecified	
Initial flour:							
Protein content (%)	9·2	9·5	8·2	8·3	6·6	6·7	8·8
Fine fraction:							
Proportion (%)	8	8	8	8	11	12	13
Protein content (%)	18·0	21·7	13·7	15·9	14·3	13·6	16·5
Intermediate fraction:							
Proportion (%)	36	37	41	34	35	26	28
Protein content (%)	5·0	3·5	3·2	3·9	2·9	2·2	3·2
Coarse fraction:							
Proportion (%)	56	55	51	60	54	62	60
Protein content (%)	10·5	11·0	9·4	9·7	7·5	7·0	9·8

Hard Wheats

Variety	Atle	Atle	Domi-nator	N.59	Mixed unspecified	
Initial flour:						
Protein content (%)	8·8	9·8	9·2	7·2	6·7	9·4
Fine fraction:						
Proportion (%)	3	5	6	4	5	5
Protein content (%)	16·6	15·2	18·6	10·8	12·1	18·6
Intermediate fraction:						
Proportion (%)	24	11	23	16	26	25
Protein content (%)	6·3	7·2	6·5	4·4	4·8	5·8
Coarse fraction:						
Proportion (%)	73	84	71	82	69	70
Protein content (%)	8·8	9·9	9·7	7·5	7·3	9·6

* Expressed on a 14 per cent moisture basis.

about double those of the initial flours. The same is true of some of the nominally hard wheat samples shown in Table III, but with those that were noticed during milling to be conspicuously hard (i.e. the N.59 and the second Atle sample—and the same effect has also been found with a Manitoba wheat not represented in Table III) the corresponding ratio is only about $1\frac{1}{2}$. Furthermore, the yield of the fine fraction from the hard wheats is much lower than that from the soft.

The yield of the intermediate fraction also tends to be lower, and that of the coarse fraction higher, with hard than with soft wheats. While with soft wheats the protein content of the intermediate fraction is generally less than half that of the initial flour, with hard the ratio is about $\frac{2}{3}$. The probable cause of the difference is that the protein matrix of the hard wheat endosperm tends during roller milling to break into larger fragments ('clusters') than that of the soft variety.

Grinding Flour before Classification

It is apparent from Table III that with soft English wheats, 50–60 per cent, and with hard, 70 per cent or more, of the flour is in the form of endosperm particles larger than about 35 μ. In these, the structure of the endosperm cell contents is more or less undisturbed. If they could be broken up after milling a further proportion of fine particles would be made available for subsequent separation into fractions of high and low protein content. The requirements of the grinding, however, are somewhat complicated. Ball-milling, for example, is capable of reducing flour particles very finely but the starch granules are thereby extensively shattered. This effect would result in spoiling of baking quality; moreover the protein in the air-classified fine fraction would be diluted with fragments of broken starch granules. On the other hand, an appropriate grinding process should reduce the protein matrix as far as possible into particles smaller than the lenticular starch granules (which it should liberate simultaneously as intact entities). Otherwise, the matrix may merely enter the intermediate fraction, in the form of 'clusters', and so prevent any marked reduction in protein content of this fraction.

Impact grinding has for some time been applied with the

object[3] of fining down flour wholly to particles under about 50 μ, an effect which it can accomplish without serious injury to starch granules. In the ordinary way, however, it does not greatly increase the proportion of material finer than about 28 μ.

Fluid Energy Mills

Fluid energy mills, such as the Micronizer,[1] are capable of disintegrating the endosperm mass very extensively—without rupturing the starch granules to a serious extent—but only, in our experience, under conditions of operation involving excessive power consumption. Some results obtained under such conditions are, however, interesting from the theoretical standpoint and are given in Table IV. This shows the effect of an 8-in. Micronizer (supplied with air at 120 lb/in²) working on an English and on an imported Canadian flour, at very small rates of feed. In each case a sample of the ground product was separated by means of the Bahco analyser into a series of fractions, the proportion and protein contents of which are shown in Table IV. Unfortunately, a

Table IV. Proportions and protein contents of fractions of various particle sizes in English and Canadian flour after treatment in a Micronizer

(Bahco separation)

Cut μ	English*				Canadian*	
	feed		grind		grind	
	proportion %	protein content %	proportion %	protein content %	proportion %	protein content %
(0–5)	—	—	(trace)	(25·8)	(trace)	(28·7)
0–13	3·9	16·3	13·5	17·3	20·1	18·5
13–17	5·6	13·6	19·8	13·5	19·4	14·5
17–22	10·7	6·7	31·0	6·4	32·2	8·0
22–28	20·5	4·7	26·2	3·2	14·6	6·4
28–35	8·2	6·9	6·2	2·6	4·9	8·4
over 35	51·1	10·2	3·3	8·9	8·8	13·8
0–17	9·5	14·6	33·3	15·0	39·5	16·5
17–35	39·4	5·7	63·4	4·7	51·7	7·6

* Initial protein contents: English 8·9 per cent; Canadian 12·4 per cent.

sample of the untreated Canadian flour was not analysed, but this happens to be of little consequence for it is clear from the Table that the Canadian flour has been no less extensively broken down than has the English.

It will be noted, with the English flour, that the differentiation in respect of protein content between the fine and the intermediate particle sizes is greater with the ground than with the initial flour. Even so, a certain proportion of very fine protein particles was lost under the conditions of these grinding tests, since the average protein content of the grind was 0·5 per cent lower than that of the feed. Quite evidently, with both the English and the Canadian flours, the Micronizer has in large measure shattered finely the protein matrix in which the small starch granules are embedded.

With the English flour, the combination of the grinding and the air classification has resolved practically the whole of the flour into one-third of high and two-thirds of low protein content. With the Canadian flour the proportion of high protein flour is (naturally, in view of the higher initial protein content) still higher, but the differentiation in protein content between the various fractions is not so great.

Despite this intensive action the grinding did not seriously damage the starch granules. Moreover the result stated is obtained with only one air-classifying operation following the grinding, whereas without the grinding two classifiers in series are required to divide flour into substantial proportions of high and low protein fractions and even then the flour treated must be of a suitable type. With the fluid energy mill, operating as in the tests of Table IV, the result is virtually independent of the type of flour used.

Pinned Disc Grinders

Apparatus exists in which to some extent the action of a fluid energy mill is obtained by direct mechanical drive. An example is the pinned disc or peg mill consisting of a housing with two parallel discs of which one is fixed while the other rotates. Both carry on their opposed faces a large number of pegs which interdigitate so that the feed, which is led to the centre of the space between the discs, must pass between them under the action of

centrifugal force. When operated at a suitably high speed, the effect of the mill is undoubtedly due less to metallic friction or impact than to the influence of alternating air disturbance on the particles traversing the orbits of the pegs. HANSSEN and FLORIAN [5] have given results obtained by means of such apparatus which support those claimed by British workers [2] and are in agreement with our own experience.

Table V shows results which we have found to be typical of the

Table V. Proportions and properties of the three main fractions of flour from Cappelle Desprez wheat, before and after pinned disc grinding

Ground or Unground	Fraction	Proportion %	Moisture content %	Protein content* %	Maltose figure† %	Colour grade value‡ units
Unground	Initial flour	100	11·9	8·3	1·2	3·6
	Fine	9	9·2	1 ·9	2·6	8·9
	Intermediate	34	10·7	3·9	1·8	2·2
	Coarse	57	11·9	9·7	0·7	1·7
Ground	Initial flour	100	10·6	8·3	1·4	3·8
	Fine	20	10·7	18·8	2·1	7·3
	Intermediate	68	12·7	5·7	1·8	2·2
	Coarse	12	12·4	8·9	1·0	6·3

* On 14 per cent moisture basis.
† Blish and Sandstedt procedure.
‡ Determined using Kent-Jones & Martin's Colour Grader. The higher the value, the poorer the colour.

effect of the grinder on soft English flours. The grinding has, approximately, doubled the proportion of both the fine and intermediate fractions and has reduced the proportion of endosperm cell fragments to the low figure of 12 per cent. Moreover, the protein content of the augmented fine fraction has been raised as a result of the entry of fragments of interstitial protein derived from the more proteinaceous pieces of endosperm which constitute initially the coarse fraction. The shortcoming, such as it is, of the grinder is reflected in the slightly raised protein content of the intermediate fraction. This suggests that not all the protein matrix has been shattered sufficiently finely to enter the fine

fraction; accordingly it contributes, in the form of 'clusters', to the intermediate fraction to an undue extent. If the matrix had been shattered to a greater degree, the yield of the fine fraction would have shown a still greater rise—towards the level of 33 per cent, already put forward in Table IV as characteristic of the maximum effect of the fluid energy mill. In this connection one must bear in mind that the protein values shown in Table IV for the fine fraction from the ground flour are doubtless low by 3 or 4 per cent through loss of very fine proteinaceous material both during operation of the Micronizer and of the Bahco Analyser.

Nevertheless, the figures in Table V show an effect due to the pinned disc mill which from the practical point of view is well worth while. Unfortunately, the effect on harder wheats appears to be somewhat less marked, and is being further studied. The figures other than those for protein in Table V will be discussed presently.

Utilization of Products

Wichser[12] has reported that American-milled flours yield the following fractions by air classification:

Description of fraction	Proportion in flour milled from: soft winter %	hard winter wheat %
1. Fine high-protein material	20	15
2. Fine low-protein material	60	25
3. Endosperm chunk particles	20*	60*

* Not stated by Wichser but estimated by difference.

No values are given for particle sizes contained in the respective fractions. If it is assumed that the cuts were made at about 17 and 35 μ, as in our own work, and that, as reported, the flour was 'natural' (not specially ground), then the contents of fractions Nos. 1 and 2 in the soft flour, and of No. 1 in the hard flour, are much higher than in European experience.

It is also stated that fractions having the following percentage

protein contents can be obtained, by grinding and air classification, from flours, initially of 11·7 per cent protein content, milled from the types of wheat mentioned above: (1) 20; (2) 6–7 (or, with a further sub-division, 3–8); (3) 11·5. While the proportions represented are not stated, the levels of protein content are in conformity with our experience. Wichser then reports that (*a*) the removal of fraction No. 2 from the hard wheat flour improves the bread-making value of the remainder; (*b*) the fraction No. 2 (from the hard wheat flour) makes fine cakes—similar to those made ordinarily from soft-wheat cake-flour; (*c*) removal of fractions Nos. 1 and 3 from a soft-wheat cake-flour much improves its cake-making quality; (*d*) while the cake-making quality of fraction No. 1 (of 20 per cent protein content) was poor, it was better than that of No. 3 (of 11·5 per cent protein content). This comparison is said to reflect the important effect of particle size on cake-making quality.

Additional Particulate Matter Separated with Fractions

Our experience so far with fractions separated, as described earlier in this report, from various English flours shows that the position regarding bread- and cake-making qualities is much more complicated than might appear from Wichser's report. In the first place, certain particulate entities other than protein fragments and starch granules occur in relatively small proportions in all flours and are distributed non-uniformly over the range of particle sizes. They therefore enter preferentially into one or other of the air-classified fractions, where they exert specific influences—additional to the effect of changes in protein level—on bread- and cake-making qualities. Moreover they are present to varying extents in different flours.

Diastatic Factors

One group of such factors is diastatic and is illustrated by the maltose figures shown in Table VI. Roughly speaking the maltose figure shown by a sample is a resultant of its contents of mechanically damaged starch granules ('ghosts') on the one hand and of β- and α-amylase on the other. In this interaction the effect of α- is greater than that of β-amylase. Normally β-amylase alone

Table VI. Instances of distribution of maltose figure among fractions separated from flours

Type of flour	Maltose figure in			
	initial flour	fine fraction	intermediate fraction	coarse fraction
1 Cappelle from Table V				
Unground	1·2	2·6	1·3	0·7
Ground	1·4	2·1	1·3	1·0
2 English of 1956 Crop	2·8	4·6	4·2	2·3
3 Dominator A	1·3	3·8	2·1	0·9
Dominator B	1·0	2·5	1·5	0·7
4 N.59 70% extraction	1·7	6·5	3·9	1·1
N.59 50% extraction	1·2	4·4	2·0	0·9

is present in flour but it may be reinforced by α-amylase resulting from sprouting caused by wet harvesting. The content of 'ghosts' in flour depends on milling conditions and, to a great extent, on the hardness of the wheat milled.[8] The form of the mechanical damage (flattening) is such that during the air classification the affected starch granules behave as though they had become smaller because their air-drag is increased, i.e. they tend to some extent to become concentrated in the fine fraction.

In Table VI, the figures for type 1, the Cappelle samples, show the case of flour milled from soft wheat, where the contents both of damaged starch and α-amylase are relatively small. Here diastatic activity is almost minimal; nevertheless it is concentrated markedly in the fine fraction. The indication of the maltose figures was supported by the results of microscopical examination, which showed that the content of 'ghosts' in the respective fractions increased progressively from coarse to fine. The 1956 crop English wheat contained sprouted grain. We do not yet know whether in such a case α-amylase is distributed uniformly throughout the fractions. The figures for type 2, English 1956 crop, suggest that it may be, and that the maltose figures of all the fractions are higher than normal as a result of the interaction of the α-amylase with the 'ghosts', which are distributed unequally among the fractions in the way just described.

The Dominator, type 3, was a harder type of English wheat. The figures for sample A could be interpreted on the basis that

while it was relatively low in amylase activity, the content of 'ghosts' was greater than in the softer wheats; the resulting marked concentration of 'ghosts' in the finer fractions therefore leads to a magnified difference in maltose figures between the fine and the coarse fractions. This conclusion is supported by the trend of the figures for sample B, relating to the same wheat which was milled specially so that the flour should contain a lower content of 'ghosts' than sample A. While the resulting difference in maltose figures of the initial flours is relatively small, owing to the concentration effect the difference becomes considerable in the finer fractions. The figures for type 4 give results on flour from N.59 wheat, the behaviour of which during milling showed it to be particularly hard for English wheat. This was laboratory-milled to two different extractions. Naturally the flour of shorter extraction (50 per cent) contained fewer 'ghosts' than that (of 70 per cent extraction) containing lower-grade streams which were excluded from the patent flour. In a sense, therefore, the comparison resembled that made with the Dominator wheat; since, however, the N.59 wheat was still harder and the content of 'ghosts' in the flour still higher, the differences of the type previously noted were intensified.

Colour

Another group of factors is that affecting colour. Flours contain small and varying amounts of finely divided discolouring matter and with air classification this is concentrated mainly in the fine fraction. This fraction has, therefore, a poorer colour than its parent flour, as may be seen from Table V.

Other Factors Affecting Baking Quality

(1) The greatest potential use for the fine fraction is for enhancing the protein content of flours deficient in protein to a level suitable for bread-making purposes. It might be expected, however, that fine fractions obtained from different wheat flours would vary in their value for this purpose since flours milled from different wheats, but having the same protein content, can vary markedly in their bread-making quality due to differences in baking strength of the protein.

The effect on bread, of differences in the quality of the protein in fine fractions separated from different flours, has been investigated by blending the fine fractions with the corresponding coarse fractions, with their parent flours and with other flours having protein contents too low to give satisfactory bread. The blends were made to have protein contents of 11 per cent, which is similar to that in normal bread flours, 12 and 13 per cent respectively. The resultant loaves, however, showed differences at any given protein level which were greater than those attributable merely to differences in protein quality.

The protein contents of the fine fractions obtained from different flours have varied, as shown in Table III, from about 10 to over 20 per cent. Thus while in some cases they were too low (under 11 per cent) for the purpose under discussion, in the other cases the amount of fine fraction required in the blend naturally varied markedly, depending on its protein content, and this resulted in large differences in diastatic activity in the blends. When this activity was low it could be increased to a suitable level by the addition of malt flour or a fungal amylase, but in many cases the activity was too high for satisfactory bread quality and this could not be corrected. This latter fault occurred particularly with those fine fractions having a high maltose figure; when those were used, diastatic activity, rather than protein quality, became the dominant factor affecting bread quality.

In those cases where the diastatic activities of blends were normal, the bread obtained was of similar quality to that given by present-day commercial bread flours, provided the fine fraction came from a flour milled from a hard wheat such as Atle. When, however, the fine fraction came from a soft wheat flour, the bread was of poorer quality, due to the crumb being more or less deficient in strength and elasticity. This could be partially rectified by increasing the percentage of fine fraction in the blend, but the benefits resulting from higher protein content were often accompanied by ill-effects resulting from increased diastatic activity.

The relative levels of protein content and diastatic activity in a fine fraction, which determine how much of it can be usefully employed in a blend, depend to some extent on the choice of wheat, since the maltose figures of a fine fraction and of its parent

flour vary in the same sense. The length of extraction of the parent flour is also of significance since diastatic activity becomes greater with increased extraction. Pre-grinding of the parent flour before classification helps since, as shown in Table V, this results in an increase in the protein content of the fine fraction without a corresponding increase in its maltose figure. The latter may in fact be reduced if the pre-grinding does not materially increase the amount of damaged starch in the flour as a whole.

The ill-effects of high diastatic activity in a fine fraction are of course most noticeable when it is blended with a weak flour of very low protein content. When blended with a more proteinaceous flour, such as might be milled from a mixed grist, the effects are diminished because of the smaller addition required. This is the direction in which a fine fraction might be most conveniently used in commercial practice, the mixed grist being rather lower in protein content than that normally used in bread flour production.

(2) The intermediate fraction, when chlorinated, has good potentialities for the manufacture of sponge and other types of cakes which have a light structure. Tests have shown, however, that the fractions separated from different flours vary in their cake-making quality, this variation being greatest with the intermediate fractions from soft and hard wheat flours.

The fractions obtained from some soft wheat flours have yielded

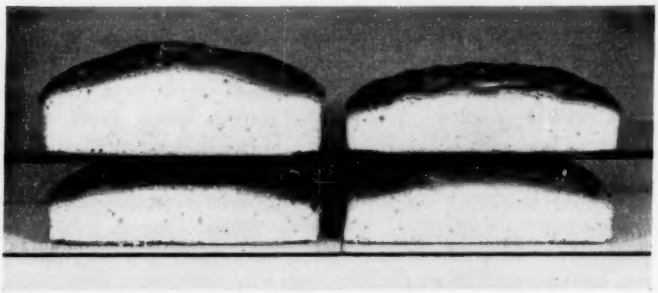

Fig. 4. Sponge cakes made from parent flours (*bottom row*) and their intermediate fractions (*top row*). Those on left from soft wheat and on right from hard wheat

excellent cakes, much superior to those made from the corresponding parent flours. On the other hand (see Fig. 4), most of the hard wheats tested have given intermediate fractions with inferior cake-making qualities. An exception was N.59, an unusual wheat in being hard yet of inferior bread-making quality. This hard wheat yielded an intermediate fraction with similar cake-making qualities to those of some, but not the better, soft wheat fractions.

Variations in the baking quality of intermediate fractions obtained from different flours appear from our tests to be due in part to variations in their contents of protein, damaged starch and amylase enzymes. We have not, however, been able to account for differences in baking quality, especially as between soft and hard wheat fractions, in terms of these factors only and it would appear that other flour characteristics are also of importance.

(3) Among the factors which determine the biscuit-making quality of a flour are: (1) the quality and quantity of its protein, which largely determine the elastic properties of the dough and the shape of biscuits baked from it; and (2) its granularity which affects the checking and tendency of biscuits to develop cracks and to break.

The application of air classification to biscuit flour production would appear to have possibilities for modifying protein content, protein quality and granularity by the elimination from or addition to the parent flour of certain fractions. Tests have shown that the elimination of the fine fraction from a flour which is too strong for biscuit-making can, by reducing its protein content, result in a dough having lower elasticity, which is desirable. On the other hand, this can be accompanied by a reduction in dough extensibility, which is undesirable. The increase in granularity due to the removal of fine material should be beneficial, and tests are in progress to determine from actual biscuit-baking trials whether this, together with the modifications to dough properties, results in better quality biscuits.

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The Protection of Some Baked Products Against Moulds by Chemical and Irradiation Techniques

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Summary. The nature of the problem of mould growth on baked products is discussed. Existing information on the reduction of this source of wastage by chemical and irradiation techniques is briefly reviewed, and new experimental evidence is presented relating to the use of these procedures. The effectiveness of ionizing radiations in preventing mould growth is indicated but there are many problems to be considered before it can be regarded as a satisfactorily established technique.

Introduction

The growth of urban populations and the increasing industrialization of large areas of the world has necessitated increasingly the ability to preserve foodstuffs for long periods against the various forms of deterioration to which they are liable. This need has been manifest in the case of the baking industry where the growing tendency to concentration of production in large units, and the correspondingly greater interval between manufacture and consumption, has increased the risk of spoilage.

There are, in general, four causes for the deterioration of baked products, namely (1) the action of bacteria (e.g. *B. subtilis*, a bacterium responsible for the development of rope in bread), (2) staling, or the occurrence of certain physicochemical changes resulting in a marked loss of palatability, (3) the development of rancidity in baked products containing fat—the rancidity may be either oxidative or lipolytic but is more frequently the former, (4) the growth of moulds. It is with the last of these that the present paper is concerned.

In the case of bread, for example, estimates made in America

and Germany put the wastage due to mould growth at 1 per cent of the annual production. Using this figure as a basis, and there is no reason to suppose that losses in this country are less than those in America and Germany, it may be calculated that some 91×10^6 lb of bread, derived from 30,000 tons of milled flour, are lost every year. Figures for wastage of cakes are more difficult to arrive at but there is no doubt that the problem is a considerable one. It should be pointed out that mould development does not constitute a problem of any significance where biscuits are concerned, because their moisture content and equilibrium humidity when packaged are so low that mould growth is impossible.

Bread is normally baked at temperatures in the region of 230–240° and cakes in the range 165–200°C. During baking the internal temperatures attained by bread and cakes are in the neighbourhood of the boiling point of water. These conditions are such that mould spores would not survive the baking process, but recontamination of the surface occurs from spores in the atmosphere of the bakery shortly afterwards; it has been estimated that mould spores settle from the atmosphere in the wrapping rooms of bakeries at a rate of 7,000–8,000 per h per m^2 of surface. Mould spores can quickly spread through a building and the danger of contamination is particularly acute when the product is sliced as the slicing blades help to spread the spores over the cut surfaces. Wrapping in waxed paper or moisture-proof film creates humidity conditions favourable to mould growth.

The equilibrium relative humidity of bread is very high (> 95 per cent). The conditions for mould development in bread are thus more favourable than in cakes, which, possessing a higher percentage of soluble solids, have equilibrium relative humidities of about 85 per cent in Madeira cakes and somewhat lower in fruited cakes.

A. The Use of Chemical Anti-mould Agents

The search for materials which would inhibit the growth of moulds dates back into the nineteenth century. One of the earliest substances found effective in this connection was vinegar, and it was used extensively by bakers for this purpose and for the prevention of bacterial development for many years.

However, more systematic work on chemicals suitable for this

purpose is of comparatively recent origin. The problem of mould growth in baked products and its control by chemical means has been discussed by TANNER[1] and more recently by PELSHENKE.[2] KIRBY, ATKIN and FREY[3] showed that fatty acids had an inhibiting effect on the growth of moulds, and HOFFMAN, SCHWEITZER and DALBY[4] established that the monocarboxylic fatty acids up to ten carbon atoms had varying degrees of effectiveness against mould, the effect being greater with increasing number of carbon atoms. These two sets of authors also indicated a specificity of action by particular acid radicals regardless of the degree of ionization of the acid. The flavour produced, however, rules out the acids containing more than three carbon atoms and since formic acid is toxic it follows that the choice is restricted to acetic and propionic acids. Later it was found that the sodium and calcium salts of propionic acid were also effective against mould.[5] It has been shown that calcium and sodium propionates are non-toxic in doses up to 3 per cent of the diet, a much larger intake than any involved in their use as anti-mould agents.[6] Benzoic acid and its derivatives have been considered as mould inhibitors; the free acid is the effective agent but since it also inhibits yeast growth this is a serious objection to its use in bread-making. The use of benzoates and related compounds in food is considered relatively harmless.[7] Sodium diacetate exhibits a mould-inhibiting effect, both through pH lowering and specific toxic action.[5] Dehydro-acetic acid has been proposed as a mould inhibitor, but although it is very efficient for the purpose it has been rejected on pharmacological grounds.

Considerable interest has been aroused recently in the use of sorbic acid as a mould inhibitor, and its efficiency in preventing mould development on cakes has been determined.[8] The mechanism by which it inhibits mould growth has been studied,[9] and it has been examined pharmacologically with the conclusion that it may safely be used in food stuffs at the appropriate levels. It is, however, of little use in fermented products since it inhibits yeast growth.

Other substances have been considered for use as anti-mould agents in baked products but for one reason or another the acceptable ones may be limited to acetic, propionic and sorbic acids, and their salts, and derivatives of benzoic acid.

Experimental Technique

Bread. The examination of the efficiency of anti-mould agents in bread was carried out as follows: A solution or suspension of the preservative of known concentration was made into a dough with white flour, salt and yeast. The basic control formula was flour 100 parts, yeast 1·8 parts, salt 1·4 parts and water 54 parts. The doughs were fermented for 3 h at 27° and baked for 30 min at 238°. The resultant 454 g loaves were exposed on a baking table overnight, during which period their surfaces became contaminated with air-borne mould spores. They were then wrapped and sealed in moisture resistant cellulose film (M.S.A.T. 300), packed into air-tight tins and stored at 27° (the optimum temperature for mould growth). All the loaves were examined daily and the time taken for mould spots to develop on each was noted. No comparisons of the density of mould growth were made. In each series of experiments there was a control dough in which no preservative was used.

The anti-mould agents examined were: propionic acid and propionates, sorbic acid and sorbates, and methyl, ethyl and propyl *p*-hydroxybenzoates.

In earlier experiments[10] the efficiency of anti-mould agents was examined by plating experiments as well as by direct incorporation into bread doughs, but in the present work the latter method has been preferred.

Cake. A typical Madeira formula was used in the cake experiments. The technique used was to prepare a cake batter from cake margarine, sugar, eggs, flour, milk and baking powder, the preservative being incorporated as a solution or suspension in milk. The batter was scaled at 0·5 kg into tins and baked at 190° for 65 min. The cakes were allowed to cool overnight on a table, during which time they became contaminated with air-borne mould spores in the same way as the bread. The procedures for wrapping, sealing, storing and examining were identical with those employed for bread, except that storage at 15° and 27° was carried out in one experiment.

For cake, the anti-mould agents examined were sorbic acid and salts of propionic acid.

Sorbic acid and calcium propionate were also compared in

cakes containing a high proportion of sugar and liquid (the so-called high-ratio cakes) since such cakes have a slightly higher equilibrium relative humidity than the Madeira cakes and are, therefore, more liable to mould development.

The levels chosen for the addition of the anti-mould agents were based on the maximum level for calcium propionate permitted for use in bread in the U.S.A. by the Food and Drugs Administration. This level is actually 0·32 per cent of the flour weight but for convenience the highest level used here was 0·35 per cent. A lower level of the order of one-half of this was also tested. In the case of the hydroxybenzoates the levels used were slightly different and were based on the recommendations of the manufacturers.

Results

Bread. The effect of the additives in prolonging storage life is summarized in Table I. They are presented in three series, since it is desirable to consider each set of experiments separately. This is because slight variations in baking, and in the extent of contamination subsequent to baking, result in a variation in the mould-free life of the controls. All of the substances examined produced some extension of mould-free life, with the exception of calcium sorbate at the levels used. The sodium hydrogen propionates* were more effective than calcium propionate, this being more noticeable at the 0·2 per cent level. 0·2 per cent propionic acid was more effective than 0·35 per cent calcium propionate. On a weight basis, sodium sorbate was also somewhat more effective than calcium propionate.

Most of the compounds studied also had a yeast inhibiting effect so that the resultant bread had a reduced volume. Calcium propionate and propionic acid (the latter in quantities not more than 0·2 per cent) had the least effect on loaf volume and had no effect on the flavour of the bread. Sorbic acid and its calcium salt inhibited yeast fermentation markedly, even in quantities as low as 0·05 per cent. The effect was so marked with sorbic acid as to preclude the making of acceptable bread. Sodium sorbate

* The sodium hydrogen propionates are complex salts formed from sodium propionate and propionic acid. The compound known commercially as sodium diacetate is similarly derived from sodium acetate and acetic acid (see ref. 5).

Table I. Mould development on bread containing mould inhibitors, at 27°

Mould inhibitor	Quantity mould inhibitor used % based on flour weight	% of loaves mouldy after days								
		3	4	5	6	7	8	9	10	11
—	0	0	0	100						
Calcium propionate	0·2	0	0	0	0	100				
„ „	0·35	0	0	0	0	0	0	60	100	
Sodium monohydrogen propionate	0·2	0	0	0	0	0	60	100		
	0·35	0	0	0	0	0	0	0	100	
Sodium di-hydrogen propionate	0·2	0	0	0	0	0	0	100		
	0·35	0	0	0	0	0	0	0	0	100
—	0	0	0	0	100					
Calcium propionate	0·35	0	0	0	30	100				
Propionic acid	0·2	0	0	0	0	30	100			
Calcium sorbate	0·05	0	0	0	100					
„ „	0·1	0	0	0	100					
—	0	0	0	0	20	100				
Calcium propionate	0·2	0	0	0	0	20	100			
„ „	0·35	0	0	0	0	0	60	100		
Sodium sorbate	0·1	0	0	0	0	80	100			
„ „	0·2	0	0	0	0	0	80	100		
—	0	0	100							
Methyl *p*-hydroxybenzoate	0·15	0	100							
„ „	0·3	0	0	0	100					
Propyl „	0·15	0	0	100						
„ „	0·3	0	0	0	50	100				
Ethyl „	0·15	0	0	100						
„ „	0·3	0	0	0	30	100				
Calcium propionate	0·35	0	0	0	0	60	100			

at levels of 0·1 and 0·2 per cent did not significantly influence bread volume but bread flavour was adversely affected. The *p*-hydroxybenzoate esters studied were also less effective than calcium propionate. At the levels used they had no adverse effect on loaf volume and flavour.

Cake. The effects of the anti-mould agents studied are summarized in Table II. The outstanding feature of these results is the marked superiority of sorbic acid over calcium propionate when compared on an equal weight basis. Only sorbic acid, and

Table II. Mould development on cakes containing mould inhibitors

Mould inhibitor	Quantity mould inhibitor used % based on flour weight	Storage temp.	% of cakes mouldy after days																			
			7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
—	0	27°	0	0	100																	
Sorbic acid	0·1	27°	0	0	0	0	0	34	34	34	100											
,, ,,	0·2	27°	0	0	0	0	0	17	17	17	100											
Calcium propionate	0·2	27°	0	0	50	50	50	100														
,, ,,	0·3	27°	0	0	68	68	68	84	84	84	100											
—	0	15°	0	0	0	0	0	0	0	0	100											
Sorbic acid	0·1	15°	0	0	0	0	0	0	0	0	17	100										
,, ,,	0·2	15°	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	84
Calcium propionate	0·2	15°	0	0	0	0	0	0	0	0	84	100										
,, ,,	0·3	15°	0	0	0	0	0	0	0	0	84	100										
—	0	27°	0	0	0	0	0	40	60	90	100											
Calcium propionate	0·2	27°	0	0	0	0	0	0	0	0	60	90	90	90	100							
,, ,,	0·35	27°	0	0	0	0	0	0	0	0	15	15	15	15	60	70	70	70	100			
Sodium monohydrogen propionate	0·2	27°	0	0	0	0	0	0	0	0	50	75	75	75	100							
	0·35	27°	0	0	0	0	0	0	0	0	10	10	10	10	50	90	90	90	90	90	90	100
Sodium dihydrogen propionate	0·2	27°	0	0	0	0	0	0	0	0	15	15	15	15	60	90	90	90	90	90	100	
	0·35	27°	0	0	0	0	0	0	0	0	0	15	15	15	15	15	15	15	25	25	25	100
—	0	27°	0	0	0	0	0	83	100													
Sorbic acid	0·1	27°	0	0	0	0	0	0	66	66	83	83	100									
,, ,,	0·2	27°	0	0	0	0	0	0	34	43	70	70	70	86	86	86	100					
,, ,,	0·3	27°	0	0	0	0	0	0	0	0	0	30	30	30	70	70	70	70	100			
Calcium propionate	0·2	27°	0	0	0	0	0	0	50	100												
,, ,,	0·3	27°	0	0	0	0	0	0	30	100												
,, ,,	0·4	27°	0	0	0	0	0	0	0	43	100											

High ratio cakes

not its salts, was studied since free acid is believed to be the effective anti-mould agent and in the case of cakes there is no problem of inhibition of fermentation. However, propionic acid was not studied in preference to its salts because it is liquid and crystalline salts are more easily handled in the bakery.

The findings at the two temperatures employed are in agreement. As was the case with bread, the hydrogen propionates are seen to be slightly superior to calcium propionate. The results with the 'high-ratio' cakes, also shown in Table II, again indicated the superiority of sorbic acid in mould inhibition.

B. The Use of Ionizing Radiations

The application of ionizing radiations to food preservation has been the subject of a great deal of research over the past decade.

Only three types of ionizing radiations seem at the moment to hold any promise for the treatment of foods. These are: (1) cathode rays; (2) soft X-rays from a suitable generator; and (3) penetrating γ rays from a radioactive source such as cobalt 60.

Radiation doses quoted in the literature are given in 'reps' or in 'rads'. The rep has never been formally defined and several different interpretations have found common use. Interpretation of results in which the dose is given in reps may, therefore, be difficult unless the unit is defined precisely. To clarify the position a new unit, the rad, has been defined which represents an energy absorption of 100 erg/g of the material being irradiated. In the following review the term rep is retained where it occurs but in the experimental work reported here the unit of dose is the rad.

There is already a very considerable volume of published work on the use and effects of ionizing radiations on foodstuffs in general. However, there appears to be very little on their application to baked products as such, although there is considerable information on the irradiation of wheat grain and flour, cake mixes and batters, whole egg and egg albumen.

Bauman *et al.*[11] irradiated white cake and spice cake mixes and found that doses of up to $0{\cdot}05 \times 10^6$ and $0{\cdot}1 \times 10^6$ rep respectively could be tolerated without serious deterioration. As is found with irradiated flour, high dose levels adversely affected starch

gelatinization. A marked deterioration of baking properties was shown by cake batters treated with doses sufficient to destroy an appreciable percentage of the bacteria present.

The possibility of irradiating baked products after they have been packaged is clearly of major importance in relation to mould inhibition. PROCTOR and KAREL[12] have shown doses of $3{\cdot}5 \times 10^6$ rep of cathode rays to produce no significant changes in the major physical properties of such materials as cellulose film, Pliofilm, polythene, glassine, Saran and waxed paper. Such high doses are unlikely to be used for wrapped baked products. LE CLAIR and COBBS[13] have concluded that irradiation levels likely to be used in food sterilization will not affect adversely various plastic materials used for food packaging.

There remains the problem of assessing any possible toxicological hazards involved when irradiated foods are eaten. This aspect necessitates much long-term research and little has been published to date. The layman's fear that irradiated foods will be radioactive can be offset by the report of MEINKE[14] who found no evidence of induced radioactivity when using a source of slightly less than 10 kilocuries of cobalt 60. The assessment of the acceptability of irradiated foodstuffs has been discussed by LEHMAN and LAUG.[15]

While ionizing radiations afford a rapid and comparatively simple method of sterilizing foodstuffs, there are problems associated with resulting flavour changes quite apart from any toxicological effects.

The spores most commonly found in bakeries are *Aspergillus niger*, *A. glaucus* and *Penicillium* species. Published figures[16–18] for the doses required to inactivate various micro-organisms indicate lethal doses for *A. niger* of $0{\cdot}34 \times 10^6$–$0{\cdot}5 \times 10^6$ rep and $0{\cdot}255 \times 10^6$–$0{\cdot}275 \times 10^6$ rep for *Penicillium*. Such figures obtained *in vitro* are not necessarily completely representative of the effects to be expected in foods but their order of magnitude is clearly such that doses sufficient to destroy the surface moulds on bread or cake would almost certainly produce some flavour deterioration. Nevertheless, lower doses might retard the development of mould whilst having no significant effect on flavour. The experimental work described below was designed to investigate this possibility.

Experimental Technique

Bread. 392 g pieces of white bread dough (formula and manufacture the same as in the preceding section) were baked in rectangular aluminium foil containers and allowed to cool overnight, permitting airborne mould contamination prior to wrapping and sealing in M.S.A.T. 300 cellulose film. The loaves remained in the foil containers during cooling and the cellulose film was applied over the containers. In this way only one surface of each loaf was contaminated and subsequently irradiated.

The irradiation levels were $0{\cdot}5 \times 10^6$, $1{\cdot}0 \times 10^6$ and $2{\cdot}0 \times 10^6$ rads, the radiation used being cathode rays from a 2 MeV Van der Graaf generator.

Storage temperatures of 27° and 15° were employed and the loaves were examined daily for mould development. In addition sample loaves were examined 24 h after irradiation for taste and smell.

Cake. In preliminary experiments small plain cup cakes, approximately 28 g in weight, were individually wrapped and sealed in moisture resistant cellulose film after being allowed to become contaminated in the same way as bread. They were then irradiated with cathode rays from a 4 MeV linear accelerator, and the dose levels used were $0{\cdot}05 \times 10^6$, $0{\cdot}1 \times 10^6$, $0{\cdot}25 \times 10^6$, $0{\cdot}5 \times 10^6$, $1{\cdot}0 \times 10^6$ and $2{\cdot}0 \times 10^6$ rads. It was found that at levels of $0{\cdot}5 \times 10^6$ rads and above marked off-flavours were immediately produced in the cakes. Mould development did not occur on cakes irradiated with $1{\cdot}0 \times 10^6$ and $2{\cdot}0 \times 10^6$ rads and only to a slight extent with $0{\cdot}5 \times 10^6$ rads; lower doses appeared to have little effect on the mould spores. It was concluded that in further experiments with cake it would be unnecessary to use doses higher than $0{\cdot}5 \times 10^6$ rads.

The systematic investigations were carried out on three types of cake in the form in which they are frequently marketed commercially:

(1) Whole plain cake; this was a typical Madeira cake formula made as described under section A. Some of the cakes were wrapped and sealed in M.S.A.T. 300 film immediately after cooling and put to one side for flavour tests after irradiation. The remainder of the cakes were left exposed overnight in the bakery to

allow normal air-borne contamination by mould spores; they were then wrapped and sealed.

(2) Sliced fruit cake; a typical rich, mixed fruit cake formula was used for this and 3 kg slabs of the batter were baked at 160°. 24 h after baking the cakes were cut into slices approximately $10 \times 10 \times 2{\cdot}5$ cm which were exposed to the atmosphere for several hours to allow contamination of the cut surfaces by air-borne spores. The slices were then wrapped and sealed in M.S.A.T. 300 film.

(3) Sliced cherry cake; a typical cherry cake formula was baked in 3 kg slabs at 160°. The finished cakes were sliced and treated in the same way as the fruit cakes.

The three types of cake were irradiated with cathode rays from a 2 MeV Van der Graaf generator at levels of $0{\cdot}1 \times 10^6$, $0{\cdot}25 \times 10^6$ and $0{\cdot}5 \times 10^6$ rads, the contaminated surface only being irradiated in each case.

After irradiation the cake slices were divided into two batches, one being stored at 27° and the other at 15°, whilst the whole cakes were stored at 27°.

The cakes were examined daily for mould growth and twice a week for organoleptic changes.

Results

Bread. The results are summarized in Table III.

Table III. Development of mould on bread treated with ionizing radiations*

Dose ($\times 10^6$ rads)	Storage temperature	% of loaves mouldy after days														
		7	8	12	13	14	19	22	27	30	33	35	37	41	51	69
0	15°	0	0	0	60	100										
0·5	15°	0	0	0	0	0	40	40	80	80	90	90	90	100		
1·0	15°	0	0	0	0	0	0	0	0	10	10	60	90	100		
2·0	15°	0	0	0	0	0	30	40	70	70	70	70	90	100		
0	27°	50	100													
0·5	27°	0	0	30	50	50	70	80	100							
1·0	27°	0	0	12	12	12	50	50	50	50	50	50	50	70	80	100
2·0	27°	0	0	12	12	12	30	30	30	50	50	50	50	80	80	100

* Examination was, as already indicated, carried out daily but the intervals given are the minimum required to show all the changes observed.

Irradiation at $0{\cdot}5 \times 10^6$ and $1{\cdot}0 \times 10^6$ rads effected a considerable extension of mould-free life. Above the $1{\cdot}0 \times 10^6$ rad level, further increase in dosage did not seem to make a significant difference. At $0{\cdot}5 \times 10^6$ rads the flavour of the bread appeared unchanged, while at $1{\cdot}0 \times 10^6$ rads there was a slight off-flavour. This off-flavour, reminiscent of fish, was quite offensive in bread irradiated at $2{\cdot}0 \times 10^6$ rads. Similar findings were obtained at both storage temperatures and, as would be expected, mould-free life was in general shortened at the higher temperature.

Cake. The results are summarized in Table IV.

Table IV. Development of mould on cakes treated with ionizing radiations**

Type of cake	Dose ($\times 10^6$ rads)	Storage temp.	% of cakes mouldy after days											
			6	11	14	18	19	20	21	26	33	41	54	69
Whole plain cake	0	27°	0	66	100									
	0·1	27°	0	0	33	55	77	77	77	*–				
	0·25	27°	0	0	0	22	22	22	22	*–				
	0·5	27°	0	0	0	0	0	11	11	*–				
Fruit cake slices	0	15°	0	0	0	0	0	0	0	0	10	22	22	77*
	0·1	15°	0	0	0	0	0	0	0	0	0	0	0	10*
	0·25	15°	0	0	0	0	0	0	0	0	0	0	0	10*
	0·5	15°	0	0	0	0	0	0	0	0	0	0	0	22*
	0	27°	0	0	0	0	10	10	10	33	44	77	77	88*
	0·1	27°	0	0	0	0	0	0	0	0	0	33	55	66*
	0·25	27°	0	0	0	0	0	0	0	0	0	0	44	44*
	0·5	27°	0	0	0	0	0	0	0	10	10	22	44	44*
Cherry cake slices	0	15°	0	0	0	0	100							
	0·1	15°	0	0	0	0	22	22	22	88	100			
	0·25	15°	0	0	0	0	0	0	0	77	100			
	0·5	15°	0	0	0	0	0	0	0	66	77	100		
	0	27°	0	0	0	0	100							
	0·1	27°	0	0	0	0	100							
	0·25	27°	0	0	0	0	100							
	0·5	27°	0	0	0	0	90	90	100					

* Examination of cakes was not continued beyond the point at which they became unpalatable due to staling.

** Examination was, as already indicated, carried out daily but the intervals given are the minimum required to show all the changes observed.

As with bread, irradiation significantly increased the mould-free shelf-life, the improvement being progressive over the range of dosage studied, i.e. $0{\cdot}1 \times 10^6$–$0{\cdot}5 \times 10^6$ rads. Similar findings were again obtained at both storage temperatures.

In plain cakes irradiation with $0{\cdot}5 \times 10^6$ rads produced an unpleasant aroma and taste which, as in bread, was reminiscent of fish. The off-flavour was only just noticeable at the $0{\cdot}25 \times 10^6$ rad level and absent at the lowest level of irradiation. Initially the off-flavour was limited to a zone extending for about 3 cm under the top crust, but subsequently it penetrated to the centre of the cake. The intensity of the irradiation flavour did not change during storage. Although the flavour bore no resemblance to the aroma and taste of oxidized fat, it was decided to determine peroxide values for some of the cakes, within 24 hours of irradiation, using the method of LEA.[19] For the plain cakes a peroxide value of 1·5 (ml of 0·002N $Na_2S_2O_3$ per g extracted fat) was obtained irrespective of the level of irradiation, which appears to confirm the idea that the off-flavour is not due to fat oxidation.

The fruit cake slices possessed no off-flavour even at the upper level of irradiation, but it is possible that the aroma and flavour of the fruit masked irradiation changes. The cherry cake slices had a very slight irradiation flavour at $0{\cdot}5 \times 10^6$ rads only. It was observed that the glacé cherries used in the cherry-cake were very slightly bleached by the ionizing radiations.

Conclusions

All the substances studied produced in bread some extension of mould-free life except calcium sorbate at the levels studied. The use of sorbic acid and sorbates in bread is precluded by their inhibition of yeast fermentation. This effect was less marked with sodium sorbate, but the flavour produced was undesirable. The propionates appear to be satisfactory anti-mould agents for use in bread, propionic acid being more effective than calcium propionate on a weight basis. The sodium hydrogen propionates were in general more effective than calcium propionate, and gave marked increases in shelf-life at the 0·2 per cent level. The dihydrogen propionate was more effective than the mono-compound, in line with the greater efficiency of free propionic acid over the calcium salt. At the levels used neither the propionates nor propionic acid produced an adverse flavour in bread. The *p*-hydroxybenzoate esters, although possessing anti-mould properties, were in general less efficient than calcium propionate.

Where cake is concerned, the propionates have an order of efficiency similar to that in bread, and again produce useful extensions of shelf-life. Sorbic acid, which is not precluded for use in cake by its yeast inhibiting properties, is markedly better than calcium propionate, particularly in the types of cake having higher equilibrium relative humidities. No adverse flavour effects in cake were noticed with these substances.

The irradiation experiments show that the technique can produce useful increases in shelf-life. However, the extent to which this can be done by increasing dosage is limited by the development of off-flavours.

It is somewhat difficult to compare the experiments with irradiation techniques and anti-mould agents where bread is concerned because the onset of mould was much less sharp with the former than with the latter. However, at doses acceptable on flavour grounds (i.e. probably up to 10^6 rads), the extension of shelf-life was generally somewhat greater than obtainable with the chemical inhibitors which could be used.

The levels which produced off-flavours in cakes were lower than those doing so in bread, and it was observed that the critical dose in this respect depended on the type of cake irradiated. In plain cakes irradiation off-flavour was noticeable at a dose-level as low as $0{\cdot}25 \times 10^6$ rads, whereas cherry cake could tolerate $0{\cdot}5 \times 10^6$ rads and fruit cakes rather more than $0{\cdot}5 \times 10^6$ rads. Doses of $0{\cdot}1 \times 10^6$ rads, which were acceptable for plain cakes, were more effective in increasing shelf-life than calcium propionate, as in the case of bread, but the extension was less than could be obtained with sorbic acid. It is interesting to note that development of irradiation off-flavour did not correlate with the peroxide values of the fat in the cakes and that at the levels used bleaching of certain ingredients could occur.

In general, the use of ionizing radiations at levels which do not produce off-flavours gives an extension of shelf-life of the same order as that which can be obtained with chemical preservatives, though possibly somewhat greater in some cases. Certainly the method bears consideration as a means of sterilizing food products, but its utility in competition with chemical agents will undoubtedly depend on the economic factors involved.

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Microbiological Evaluation of Peptones

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Summary. Information on a reliable method for determining the nutritive value of existing and experimental peptones is lacking in the literature. To overcome this handicap, a method for comparative evaluation of microbiological peptones has been developed.

The method is based on measuring the growth of eight carefully selected, fastidious, test cultures. The cultures are grown in presence and absence of yeast extract in an inorganic basal medium containing test peptone and a low and high level of glucose.

Statistical analysis of replicate peptone evaluations of six peptones, conducted over a period of six months, shows that 95 per cent confidence limits for this method are $\pm 2{\cdot}5$ to 7·9 per cent of the mean.

Four criteria are employed for determining the overall performance of 12 widely used, commercial peptones.

The method is very sensitive for detecting differences between batches of commercial peptones. With the aid of this method a new peptone 'N-Z-Amine YT' has been developed. Considering performance and relative cost, this peptone is the most economical of the peptones evaluated.

Introduction

The main microbiological applications of peptones are for the production of antibiotics, enzymes, toxin-toxoids, vaccines, starter cultures, cortisone or its precursors, and a great variety of packaged microbiological media. Because of such diversified applications the evaluation of peptones on the basis of their end-use was discarded, especially in view of the fact that in many instances methods of production and yields of such end-products are not generally known. The extent of growth of a few carefully selected bacterial cultures, in a basal medium containing test

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peptones, was used as a yardstick for measuring the comparative efficiency of different peptones.

It is fully realized that quite often a peptone suitable for growth of an organism is not satisfactory for the production of a desired end-product by that organism. However, because of the aforementioned handicaps, the task of evaluating a peptone for a specific end-use has been left to industry.

In order to compare many peptones at the same time and under similar conditions, the evaluation procedures must be quick and dependable. The growth of test organisms was therefore measured as optical density (OD), instead of using lengthy agar plating or tube-dilution techniques. This restricted the choice of test organisms by eliminating those that form a pellicle or clump, and thus do not produce a uniform growth or turbidity required for the measurement of optical density. In general, commercial peptones produce clear solutions when used in microbiological media, and are therefore suitable for the determination of optical density, before and after the growth of a test culture in the medium.

Materials and Methods

Media

Preliminary evaluation of peptones was done in a simple basal medium. It was found that trace amounts of certain salts increased considerably the growth of a few test cultures. These salts were therefore included in the improved basal medium from which four media were derived for the comparative evaluation of peptones. The composition of these media is shown in Table I.

Table I. Composition of media used for comparative evaluation of peptones

Basal medium	Per 100 ml	L-medium	H-medium	L-medium YE	H-medium YE
NaCl	0·2 g				
K_2HPO_4	0·2 g	Basal	Basal	Basal	Basal
$MgSO_4 \cdot 7H_2O$	20·0 mg				
$FeSO_4 \cdot 7H_2O$	1·0 mg				
$MnSO_4 \cdot 4H_2O$	1·0 mg				
Glucose	—	0·25%	1·0%	0·25%	1·0%
Yeast Extract	—	—	—	0·3%	0·3%

The concentration of test peptones used in these media was always 2 per cent. All ingredients of a medium were dissolved in water, applying a little heat if necessary, pH was adjusted to 7·0, and the volume was made to 100 ml. If a medium had a haze or floc, it was filtered off prior to its distribution at 10 ml per tube into ten tubes. One drop of cysteine bicarbonate per tube, prepared by the method of HUHTANEN *et al.*,[3] was added to five tubes which were vaspar-sealed for growing anaerobic test cultures. All tubes were autoclaved at 15 lb/in^2 for 10 min.

Test Cultures

Eight organisms, four anaerobic and four facultative, were selected as test cultures for the evaluation of peptones and are shown below:

(1) L_4 Anaerobic Rumen Organism (Our Isolate)
(2) Sp " " " " "
(3) CL " " " " "
(4) An-22 *Clostridium perfringens* (Baltimore Biological Laboratory)
(5) LB-10 *Lactobacillus arabinosus* (ATCC–8014)
(6) K_1B *Streptococcus lactis* (Our Strain)
(7) Sm_1 *Leuconostoc mesenteroides* (ATCC–8042)
(8) SF_1 *Streptococcus faecalis* (ATCC–9790)

Anaerobic cultures, one to four, were carried in the culture broth of HUHTANEN *et al.*,[3] with the modification that in place of cotton one drop of 2 per cent alfalfa meal (40 mesh) suspension was added per tube. Facultative cultures, five to eight, were carried in Trypticase Soy Broth (Baltimore Biological Laboratory). All cultures were transferred twice weekly, incubated for 24 h at 37°C (except K_1B which was incubated at 27°C), and were held at room temperature between transfers.

Inoculating and Reading Culture Tubes

Only 22 ± 2 h old test cultures were used as inocula for seeding a series of 10 tubes, representing a test peptone included in one of the four media described (Table I). The inoculum was transferred into culture tubes as a small droplet with a sterile Dispo-pette (Pasteur capillary pipettes obtainable from Scientific

Products, Flushing, New York). The anaerobic test cultures were seeded into four vaspar-sealed tubes, and the facultative cultures into four nonvaspar-sealed tubes. The remaining two tubes, from the anaerobic and facultative series, were used as blanks for measuring optical density of the cultures.

All tubes were incubated at proper temperatures for 42 ± 2 h, the cultures were then shaken thoroughly so as to form a uniform turbidity and were transferred to special lumetron tubes. Test cultures Sp and An-22 produce gas, and their tubes are always read after all gas has been removed from the culture with the aid of a glass rod. Coleman Nepho-Colorimeter was employed for the determination of optical density, using 525 mμ optical filter.

Experimental Results

Selection of Test Cultures

The cultures LB-10, Sm_1 and SF_1 were selected because they are used for microbiological assay of 17 amino acids and 3 vitamins, namely, biotin, niacin and pantothenic acid.

The culture An-22 represented the pathogens as well as the toxin-toxoid producing clostridia, and K_1B represented lactic starter cultures. From the other cultures screened, three rumen

Table II. Growth (OD × 100) of various cultures in media containing different soy peptones

	Anaerobic rumen cultures (our isolates)								
Soy peptone	L_2	L_3	L_4	CR	TH	Sp	CL	HD_1	LB-10
1*	41	20	0	29	41	20	0	34	0
2*	46	21	0	24	50	28	0	30	0
5*	30	9	0	28	34	5	1	13	0
6*	30	13	0	31	37	9	0	14	0
PA-2*	23	2	0	1	11	8	7	38	9
SA-2*	22	6	0	10	31	21	0	38	6
Commercial A	41	22	2	32	37	18	1	39	32
Commercial B	41	30	0	49	52	20	3	58	46

* Prepared in the laboratory.

cultures L_4, Sp, and CL were selected because they were found to be fastidious, as evidenced by their scanty growth in a simple medium containing sodium chloride, dibasic potassium phosphate, glucose, and various soy peptones shown in Table II. Physiologically, L_4 is a homofermentative lactic culture, Sp a gas former, and CL produces fatty acids from glucose.

Significance of Glucose and the Ingredients of Basal Medium

To establish the significance and levels of various ingredients of the basal medium, eight test cultures were grown in the media shown in Fig. 1.

The variable between media A and B was additional salts, B and C additional glucose, and C and D additional phosphate. Each medium was split into three media, each containing a

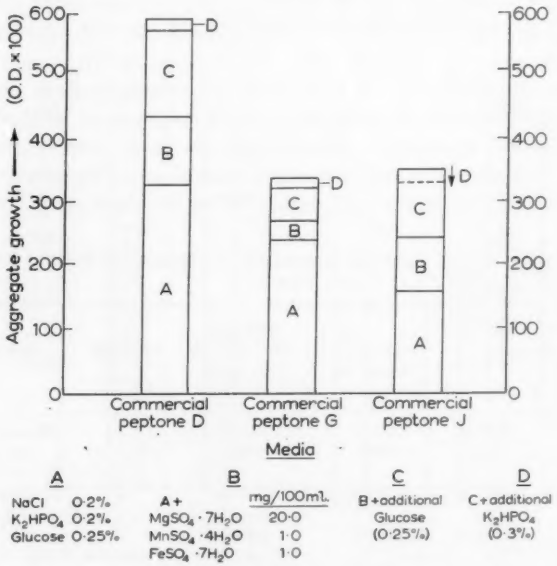

Fig. 1. Effect of additional salts, glucose and phosphate on aggregate growth of test cultures

different commercial peptone. The histograms in Fig. 1 show that increased phosphate (D) did not have any appreciable effect on aggregate growth (combined optical densities of 8 cultures) of test cultures, whereas additional salts (B) and glucose (C) increased significantly the growth of these cultures.

In another experiment, increasing the concentration of glucose from 0·5 to 1·0 per cent did not change aggregate growth of test cultures. However, when the medium was reinforced with yeast extract, 1·0 per cent glucose in the basal medium was found to be desirable.

Establishment of the Level of Yeast Extract in L- and H-media

In order to supply vitamins, growth factors, and degradation products of nucleic acid, yeast extract was used for supplementing L- or H-medium. In addition to these nutrients it supplied some amino acids, peptides and minerals. The level of yeast extract, at which it provided sufficient vitamins and growth factors, and

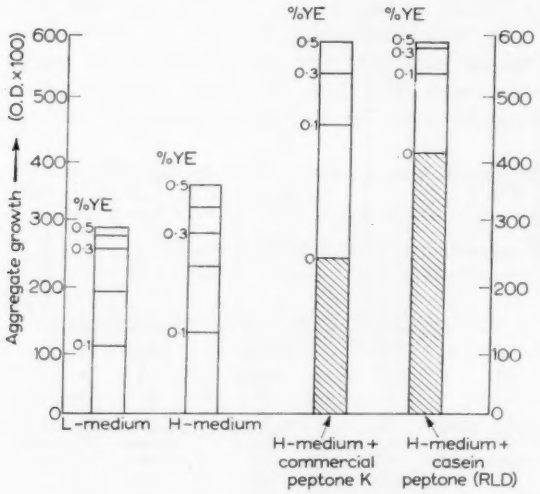

Fig. 2. Aggregate growth from ascending concentration of yeast extract (YE)

beyond which it possibly became just a source of nitrogen for test cultures, was determined as follows:

Difco yeast extract was added to L- and H-media at concentrations 0·1, 0·2, 0·3, 0·4, and 0·5 per cent and the growth of test organisms was determined both in absence and presence of a peptone. The results are shown in Fig. 2.

The histograms in Fig. 2 show that aggregate growth dropped very considerably beyond the level of 0·3 per cent yeast extract in L- and H-media, both in presence and absence of a peptone. It was thus established that 0·3 per cent yeast extract in L- and H-media should be adequate to supply necessary vitamins and growth factors for most of the test cultures.

Reproducibility of Results

Reproducibility of results obtained by this method was checked by using duplicate and replicate sets of experiments. The growth of test cultures was determined in duplicate in six media, each containing a different peptone. The average per cent difference between duplicates was found to be 1·8. In the replicate set of experiments, each peptone was evaluated at three different times within a period of about six months. Standard deviation and 95 per cent confidence limits for the mean were determined on data obtained from replicate experiments, and are shown in Table III.

Table III. Statistical analysis of variation in aggregate growth using replicate tests

Peptone	Medium	Aggregate growth (OD) mean of 3 replicates	Standard deviation ±	95% Confidence limits ±	
				the mean	% of the mean
Commercial N	L	3·89	0·099	0·245	6·3
Soy peptone WYN*	L	3·25	0·032	0·081	2·5
Commercial O	L	2·90	0·062	0·155	5·3
Lactalbumin peptone WYN*	L	2·73	0·070	0·174	6·4
Commercial K	H	2·49	0·053	0·131	5·3
Commercial F	H	4·04	0·125	0·310	7·9

* Peptones prepared in the laboratory.

The data show that the method is precise, and reproducibility is good for both replicate and duplicate tests. Variations in replicate experiments are small, although not as small as in duplicate tests. In order to reduce variation caused by replication, comparative evaluation of peptones by the present method is always conducted simultaneously. The sensitivity of the method is checked from time to time by evaluating a peptone in H-medium, and determining whether the aggregate growth from it lies within 95 per cent confidence limits, established for it previously.

Criteria for Comparative Evaluation of Peptones

The comparative evaluation of commercial peptones was based on their overall performance with respect to the following, pre-established, four criteria:

(1) Aggregate growth in H-medium.
(2) Aggregate growth from increased glucose (0·75 per cent), determined by subtracting aggregate growth of L-medium from that of H-medium.
(3) Aggregate growth in H-medium YE.
(4) Aggregate growth from increased glucose (0·75 per cent), determined by subtracting aggregate growth of L-medium YE from that of H-medium YE.

Comparative Evaluation of Microbiological Peptones

After a preliminary survey of the literature,[1, 2] 11 most widely used commercial peptones and a casein peptone, developed with the aid of this method, and available commercially as 'N-Z-Amine YT' from Sheffield Chemical Division, Norwich, New York, were selected. The peptones were Proteose-peptone, Bacto-peptone, Tryptose, Trypticase, Thiotone, Gelysate, Soy Peptone, Meat Peptone, 'N-Z-Amine A' (a pancreatic digest of casein), 'N-Z-Amine E' (a tryptic digest of casein), and 'N-Z-Amine YT'. In the comparative evaluation of these peptones, only 'N-Z-Amine YT' and Soy Peptone are identified, and the rest of the peptones are given a code letter. All peptones were tested for fermentable carbohydrate[6] and total reducing substances,[5] and were found to be appreciably free from both, except Soy Peptone which contained substantial amounts of fermentable carbohydrate. Accurate evaluation of Soy Peptone by this method is, therefore, not possible.

Each test peptone was incorporated at 2 per cent into four media, namely, L-medium, H-medium, L-medium YE, and H-medium YE. The individual and aggregate growths of eight cultures in these media are shown in Tables IV, V, VI, and VII. Each value represents an average of two replicate evaluations.

In general, anaerobic cultures did not grow as well as the facultative cultures in L- and H-media (Tables IV and V). The

Table IV. Growth (OD × 100) of test cultures in L-medium

Peptone	Test cultures								Aggregate growth
	L_4	Sp	CL	An-22	LB-10	K_1B	Sm_1	SF_1	
Commercial D	42	44	50	35	61	43	55	47	377
„ E	41	27	67	16	54	35	22	27	289
N-Z-amine YT	32	29	65	22	44	43	20	27	282
Commercial F	32	27	57	18	49	38	15	24	260
„ C	38	7	71	12	55	32	17	24	256
Soy peptone*	33	40	11	25	39	38	7	39	232
Commercial J	10	10	35	18	47	40	27	34	221
„ G	3	11	48	28	28	39	26	27	210
„ H	16	7	27	24	40	23	24	31	192
„ K	12	8	28	20	29	41	15	28	181
„ M	19	2	8	12	17	30	13	22	123
„ L	17	6	4	11	20	25	13	18	114

* No glucose added to the medium, as Soy Peptone contained fermentable carbohydrate.

Table V. Growth (OD × 100) of test cultures in H-medium

Peptone	Test cultures								Aggregate growth
	L_4	Sp	CL	An-22	LB-10	K_1B	Sm_1	SF_1	
Commercial D	75	83	85	82	71	46	93	50	585
Soy peptone	70	49	89	43	86	53	65	64	519
Commercial C	86	4	90	28	85	51	110	64	518
„ E	85	39	51	59	72	47	91	61	505
N-Z-amine YT	89	53	55	46	73	53	80	45	494
Commercial F	69	32	30	42	71	52	63	58	417
„ J	28	8	22	30	60	40	77	46	311
„ H	39	14	20	33	61	24	59	37	287
„ G	9	11	47	33	26	49	37	33	245
„ K	26	6	18	23	28	47	17	28	193
„ L	30	3	4	16	20	25	13	18	129
„ M	31	1	3	10	20	29	11	20	125

scanty growth (underlined values) of cultures, except Sm_1, did not increase from L- to H-medium in certain peptones, showing that substances other than glucose were the limiting factors. Addition of 0·3 per cent yeast extract increased the growth of all these cultures substantially (Tables VI and VII). However, culture Sm_1 hardly grew in L-medium and L-medium YE, but

Table VI. Growth (OD × 100) of test cultures in L-medium YE

Peptone	Test cultures								Aggregate growth
	L_4	Sp	CL	An-22	LB-10	K_1B	Sm_1	SF_1	
Commercial D	50	49	68	30	58	39	46	44	384
,, L	38	40	59	31	49	36	26	32	311
,, J	47	47	72	23	47	38	12	23	309
N-Z-amine YT	44	42	78	24	47	40	14	16	305
Commercial K	43	39	76	22	41	41	22	20	304
,, E	49	43	74	20	52	28	16	17	299
,, G	32	38	72	26	32	36	24	31	291
,, C	45	46	77	15	49	24	17	16	289
,, H	43	36	73	21	45	37	12	17	284
Soy peptone*	55	47	17	31	43	37	8	34	272
Commercial M	41	35	34	31	44	36	25	24	270
,, F	41	37	73	17	39	29	15	14	265

* No glucose added to the medium.

Table VII. Growth (OD × 100) of test cultures in H-medium YE

Peptone	Test cultures								Aggregate growth
	L_4	Sp	CL	An-22	LB-10	K_1B	Sm_1	SF_1	
Soy peptone	114	94	99	65	101	58	99	71	701
Commercial C	84	88	114	51	87	45	118	62	649
,, D	86	95	100	82	79	46	98	59	645
N-Z-amine YT	89	90	115	62	87	44	91	56	634
Commercial E	88	94	88	74	82	46	95	62	629
,, G	91	80	118	50	78	46	85	60	605
,, F	72	86	72	72	84	47	89	65	587
,, K	76	76	108	46	79	41	79	54	559
,, H	54	81	95	46	83	41	84	55	539
,, J	42	78	81	40	79	41	84	58	503
,, L	47	63	65	60	71	38	80	45	469
,, M	43	53	49	40	70	35	77	44	411

grew well in the other two media, showing that in this case glucose was the limiting factor.

Based on four criteria, the final evaluation of the 12 test peptones is shown in Table VIII. The peptones are given in order of descending overall performance. Peptones, which are enclosed within brackets, do not differ from each other significantly in performance.

Table VIII. Comparative performance of test peptones based on four criteria

Peptone	Aggregate growth (OD × 100) for criteria				Sum of aggregate growths	Relative cost
	(1) H-medium	(2) increased glucose (0·75%)	(3) H-medium YE	(4) increased glucose (0·75%)		
Soy peptone*	519	287	701	429	1,936	1·4
Commercial C	518	262	649	360	1,789	2·6
„ D	585	208	645	261	1,699	3·8
„ E	505	216	629	330	1,680	3·8
N-Z-amine YT	494	212	634	329	1,669	1·0
Commercial F	417	157	587	322	1,483	2·6
„ G	245	35	605	314	1,199	2·2
„ H	287	95	539	255	1,176	0·9
„ J	311	90	503	194	1,098	3·1
„ K	193	12	559	255	1,019	0·8
„ L	129	15	469	158	771	2·7
„ M	125	2	411	141	679	1·8
Basal + Yeast extract (no peptone)			290	23		

* Soy peptone values are boxed because they could not be evaluated accurately by this method.

Using 'N-Z-Amine YT' as a unit, the relative cost of a peptone was derived from the price per pound of the peptone per 48 to 175 pound lot. Taking cost and performance into consideration, 'N-Z-Amine YT' was found to be the most economical of the peptones evaluated.

Poor performance of peptones G and K (Table VIII) for criterion two, and a good score for criterion four, show that these peptones are possibly deficient in vitamins, growth factors, and purine-pyrimidine compounds, but are a good source of peptides and

amino acids. However, poor showing of peptones L and M with respect to criterion four, indicates inferiority, which may be due to a lack of certain amino acid(s) and peptide(s), or the presence of certain growth inhibitors[7] for the test cultures.

Discussion

Peptones are such complex materials derived from natural substances that a foolproof method for the evaluation of all peptones will be impossible to develop. Besides, the methods and materials used for their production are trade secrets and it is possible that some peptones are fortified with substances that are foreign to them.

The method described above is reproducible, thorough, and practical, and has been under development and scrutiny for about two years. It determines the nutritive value of a peptone for supporting the growth of micro-organisms, but it does not measure the products of their metabolism.[4]

The eight test cultures used are fastidious and require a variety of nutrients. A peptone which supports good growth of all these cultures will undoubtedly be suitable for microbiological media. The method is a valuable tool for detecting variations between batches of peptones, for checking out intended modifications in the production processes, and for evaluating the effect on nutritive value of a peptone of such processing practices as heating and charcoal treatment.

In the search to find more nutritious peptones, the method has been employed routinely for evaluating over 230 experimental protein hydrolysates. It has been of considerable value in determining the performance of peptones prepared in the laboratory from such diversified, high-protein materials as extracted soy meal, peanut meal, castor pomace, guar protein, feather meal, fish meal, tankage, meat scraps, blood meal, torula yeast, brewer's yeast, corn gluten, soy protein, egg white, egg yolk, casein, and lactalbumin, using proteolytic enzymes of plant, animal, and microbial origin.

The method has been the most important single factor in the development of the new peptone 'N-Z-Amine YT'.

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